Exosomal miR-24-2 in Mediating Mitral Valve Dysfunction by Elevation of TGF-B Levels during Rheumatic Heart Disease Pathogenesis

Shruti Sharma[1], Harkant Singh[2], Seema Chopra[3], Uma Nahar[4], Anuradha Chakraborti[5]*

[1, 5] Experimental Medicine and Biotechnology,
Postgraduate Institute of Medical Education and Research, Chandigarh, India
[2] Cardiothoracic and Vascular Surgery,
Postgraduate Institute of Medical Education and Research, Chandigarh, India
[3] Obstetrics & Gynecology,
Postgraduate Institute of Medical Education and Research, Chandigarh, India
[4] Histopathology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Email: [1] chakravarti.anuradha@pgimer.edu.in, [2] hrutisharma.8722@gmail.com, [3] singh.harkant@pgimer.edu.in, [4] chopra.seema@pgimer.edu.in, [5] nahar.uma@pgimer.edu.in

Abstract

Rheumatic heart disease (RHD) is an autoimmune sequel of rheumatic fever (RF) that results in permanent damage to the mitral valves of the heart. Endothelial cells (ECs) of the mitral valves, take up exosomes in the biological fluids which may change the cellular characteristics of the cells. Exosomal miRNAs, have been known to play an imminent role in various diseases. The composition of exosomal miRNAs, varies, under different physiological and pathological conditions. Herein, an attempt has been made to unfurl the role of exosomal miRNA, miR-24-2 in mitral valves damage during RHD. The present study is focused on *in-vitro* set up and patient samples analysis in which the exosomes were isolated from patient samples (serum and pericardial fluid) of RHD patients and simultaneously from the media extracted RHD patients' serum stimulated HUVECs (RHD-*in-vitro* model). Next, HUVECs were, treated with exosomes (component of serum) from RHD patient's serum (R-exo) and normal individuals' serum (N-exo) in-order to evaluate whether HUVECs treated with exosomes brings about morphological changes in HUVECs and change in biological properties. Further, miRNA profiling and RT-PCR of the isolated exosomal RNA revealed that miR-24-2 level was attenuated in RHD patients' samples as well as in the media. Finally, to check cause and effect of the study, functional inhibition of exosomal miR-24-2 was performed, in exosomes treated HUVECs. Decreased expression levels of miR-24-2 as well as endothelial marker (VE cadherin) and increased levels of mesenchymal (SMA-α) and fibrosis marker (TGF-β) was observed in R-exo-treated HUVECs in comparison to N-exo treated HUVECs, which was correlated with altered proteins levels of these markers in the mitral valves of RHD patients. Thus, these findings suggests, decreased exosomal miR-24-2 levels may lead to enhanced TGF-β production and mitral valves dysfunction (stenosis and regurgitation) during RHD.

Keywords: Rheumatic heart disease; exosomes; microRNA; Fibrosis; EndoMT,

1. Introduction

Rheumatic heart disease (RHD) remains a serious health problem in low-and middle-income countries and is a cause for high morbidity and mortality worldwide. The disease, consequently, results in permanent mitral valves damage, caused due to recurrent events of rheumatic fever (RF), an autoimmune inflammatory reaction against infection in throat caused by group A streptococcus (GAS) **[1].** It occurs mostly in children and may lead to life-long disability or death**.** According to WHO report, 2018, RHD affected 30 million people globally, in 2015, and caused 305000 deaths and 11.5 million disability-adjusted life years lost. Molecular mimicry theory (MMT) is the mechanism responsible for RHD pathogenesis. According to this theory, there exists a structural similarity between Group A streptococcus (GAS) proteins and cardiac proteins, hence, auto-antibodies produced against GAS, cross-reacts with cardiac proteins and attacks the heart valve endothelium, that leads to increased expression of cell adhesion molecules like vascular cell adhesion molecule-1 (vCAM-1) and iCAM (intercellular cell adhesion molecule). The cellular infiltration in the mitral valves leads to inflammation, that results in initially, scarring in the mitral valves and eventually RHD **[2].** Fibroblasts (mesenchymal origin) which differentiates into myo-fibroblasts are responsible for tissue fibrosis, also diseased conditions leads to the release of cytokines by fibroblasts such as TGF-β, which enhances fibrosis during pathological conditions.**[3].**

Mitral valves fibrosis is the hallmark of RHD, which leads to mitral valves dysfunction **[4].**It is therefore important to unveil the causative factors which leads to mitral valves fibrosis during RHD. Now a days, research has been focussed on exosomes, which play an important role in various physiological and pathological cardiovascular processes **[5].** Exosomes are released by different cell types **[6]**, found in various body fluids such as serum, plasma, breast milk, saliva, amniotic fluid, urine and also in cell culture media **[7],** and are abundant in bio-molecules, such as, RNAs, microRNAs (miRNAs), DNAs, lipids and proteins. In addition, by transfer of these bioactive cargos to recipient cells, these vesicles act as cell to cell communicators and exosomal miRNAs have proven to play an imminent role in promoting or preventing various diseases.

MicroRNAs are small non-coding RNAs which are transcribed first as primary miRNAs (pri-miRNAs) from DNA sequences and transformed into mature miRNAs via precursor miRNAs (pre-miRNAs), with an average of 22 nucleotides in length. The miRNAs interfere with the 3' UTR of target mRNAs in most cases to reduce the expression of target mRNAs **[8].** However, interactions between miRNAs and other regions have also been documented, including the 5' UTR, coding sequence, and gene promoters **[9].** Under some situations, miRNAs have been shown to stimulate gene expression.**[10].** Recent research has shown that miRNAs are transferred between various compartments of the cell to regulate the translation rate as well as transcription **[11].**

Various studies have indicated that miR-24-2 is known to play a role in EndoMT and fibrosis during various cardiac diseases **[12,13].** However, the role of exosomal miRNA-24-2 in RHD is not yet unfurled. Hence, in the present study, mitral valve damage by exosomal miRNA-24-2 has been investigated.

2. Materials and Methods

Collection and processing of samples

The present study was ethically approved by Institutional Ethics Committee (INT/IEC/2017/1453). Patient confidentiality was maintained throughout the study with strict adherence to guidelines stipulated by IEC and informed consent was taken from each participant. A total of 42 clinically detected RHD patients were included in the present study. Mitral valve tissues, blood and pericardial fluid samples were collected from these patients while undergoing mitral valve replacement surgery (MVR) and double valve replacement (DVR) in the department of Cardiothoracic and Vascular Surgery (CTVS), Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Pericardial fluid samples (as controls) were collected from non RHD patients, undergoing coronary artery bypass graft (CABGs), from the same department. For control, blood samples were obtained from healthy individuals and mitral valve tissues were collected from cadavers with no pathological or morphological heart disease. Umbilical cords (n=10) were collected from the department of Obstetrics and Gynaecology from full-term healthy mothers and processed on the same day. The patients suffering from any autoimmune disease, or any infectious disease and not willing to give consent were excluded from the study. During processing, mitral valve tissues (100-150mg) were collected in PBS and brought to the laboratory in ice. The tissues were finely minced and kept overnight in RNA later™ (Thermofisher Scientific, USA) at 4°C, and then shifted to -80°C in RNA later till further use. Serum was separated and 1X protease inhibitor (Sigma-Aldrich, USA) was added and stored at -80°C until further use. After removal of debris from PF, the samples were centrifuged at 4000g for 15 min and were stored at -80°C until further use.

Isolation, culture and characterization of Human Umbilical Vein Endothelial cells (HUVECs)

HUVECs were isolated from human umbilical cord **[14].** Briefly, the cord was brought to the laboratory in 1X PBS containing penicillin (1million unit/50ml) at 4°C. The cannula was inserted in the vein and washed with PBS once. 0.2% Collagenase (Gibco, USA) was injected from one end and the cord was clamped from the other end. The cord was kept at 37 °C for 30 min, and collagenase was collected in a fresh tube. One wash with EGM-2 media was given and effluent media, pooled with collagenase was centrifuged at 1500 rpm for 3 min to pellet down the cells. The pellet was washed with endothelial cell growth medium (Sigma Aldrich, USA) and plated on 6 well plate (Corning, USA) at 37°C in 5% CO_2 incubator. Cultured cells were trypsinized (Lonza, Switzerland) and centrifuged at 1200 rpm for 3 min and the cell pellet was suspended in a fresh EBM-2 medium containing growth factors. Cell count was determined using Neubauer chamber (Marinfeld, Germany) and thereafter seeded on 96 well plate. HUVECs were grown on 96 well plates till 50-60% confluency. Fixing of cells were carried out with 4% paraformaldehyde in phosphate-buffered saline (PBS) at RT followed by two washes with PBS and blocking with 5% BSA. The wash step was repeated and incubated with anti CD-31 antibody (Sigma-Aldrich, USA) at 37°C and then washed twice with PBS to remove the unbound antibody. Secondary anti-human IgG labeled with phycoerythrin (PE) (Santacruz Biotechnology, USA) was added and incubated at 37°C.

Nuclei were stained with DAPI stain (Sigma-Aldrich, USA). Cells were visualized in the EVOS cell imaging system (Thermofischer, USA.

Stimulation of HUVECs with RHD patients' pooled serum to develop *in-vitro* RHD model

Cells were grown to 70-80% confluency and RHD patients' pooled serum was added to the cells in 12 well plate to the final dilution of 1:5, 1:50, and 1:100 for 6h and 12h in 12 well plate. Cells were microscopically viewed and documented in EVOS FL Auto (Life technologies, USA). Further, RNA isolation was carried out from cells by TRIZOL reagent (Invitrogen, USA). cDNA was synthesized from isolated RNA and related gene expression of the genes (INF-γ, TNF-α, IN-1β and vCAM) involved during RHD pathogenesis was carried out to confirm the development of RHD *in-vitro* model **[15].** For collection of media, HUVECs were cultured at 90-100 %, density in medium with 10% exosomes-depleted FBS (Gibco, USA) for 72 hr. The medium was centrifuged for 10 min at 2,000 x g, then for 30 min at 10,000 x g at 4 C. The supernatant was filtered through a 0.22 μm filter and concentrated using centrifugal ultrafiltration (Amicon® Ultra-15 100 KDa; Merck KGaA) to eliminate cellular debris.

Exosomes isolation

Exosomes were isolated from processed media and biological fluids (serum and PF) with miRCury exosomes isolation kit (Qiagen, Germany), according to the manufacturer's instructions. Briefly, the media as well as biological fluids were mixed with exosomes precipitation reagent at a ratio of 4:1 followed by overnight incubation at 4˚C. Further, centrifugation was carried out at 500 x g for 5 min for serum and 10,000 x g for 30 min for PF and media. Next, exosomes pellets were resuspended in PBS (GE Healthcare Life Sciences, USA). The exosomes pellets were used at once or stored at -80˚C till further use.

Transmission Electron Microscopy (TEM)

TEM was used to examine the exosomes morphology. Briefly, 20 μl of exosomes-PBS solution at 1:40 dilution was poured onto carbon-coated copper grids and allowed to stand for 1 min. Exosomes were stained with 20 μl of uranyl acetate dihydrate (2%) and left for 1 min. The sample was air dried for 10 min and viewed under transmission electron microscope (H7650 Hitachi, Japan).

Zeta sizing

Zeta sizer (Malvern paranalytical, UK, NIPER, Mohali) was used to measure the particle size diameter and percentage intensity of exosomes using the technique of Dynamic Light Scattering (DLS), according to the operating instruction. The sample was prepared by dispersing exosomes in PBS at 1:400 dilution.

Western blot

HUVECs and exosomes were lysed in ice cold RIPA buffer. The measurement of protein concentration was done by BCA method. The protein samples (50 μg) were loaded on to SDS-PAGE and the protein bands from the gel were transferred (80V, 2.5h, 4ºC) onto Polyvinylidene fluoride membrane (PVDF) (GE Health care, USA) in the transfer buffer [25mM Tris/192mM Glycine (pH 8.0)/20% methanol] using mini-tank transfer unit (Bio-Rad mini Transblot® Cell, Bio-Rad, USA). After transfer, the blocking of membrane was done with 5% skimmed milk in Tris-buffered saline-0.1% tween 20 (TBST overnight at 4ºC). The

membrane was washed thrice for 5 min each with TBST to remove unbound blocking reagent. Primary antibody against CD- 63, (Biovision, USA, 1:100) SMA-α, (Biovision, USA, 1:200) TGF- β (Biovision, USA, 1:200), VE cadherin (Biovision, USA, 1:100) and β-actin,(Biovision, USA, 1:200) was added and incubated at 37ºC with shaking. Washing steps were repeated. Further, the membrane was incubated with secondary antibody (Biovision, USA, 1:2000), solution (HRP conjugated, Bangalore Genei, India) at 37ºC with shaking. Again, washing steps were repeated. Membrane was exposed for 1 min or longer for visualization and images were acquired in Protein Simple FlourChem M (Bio-Techne, USA). Densitometric quantification of bands was done using image J.

Exosomes uptake in HUVECs

In-order to confirm exosomes uptake by HUVECs, exosomes uptake experiment was performed by labelling exosomes with PKH-67 dye (Sigma, USA), according to manufacturer's instructions. The dye labels the lipophilic exosomal membrane. Briefly, PKH-67 dye (2 µl) was diluted in 100 µl diluent C and incubated at 37°C for 15 min. Exosomes (10 µl) was dissolved in 20 µl PBS and 80 µl of diluent C was added to the dye and incubated for 5 min while mixing. Next, 100 µl of 10% exosomes-depleted FBS was added & the mixture was filtered with amicon filters 10kDa (Merck, USA). Labelled exosomes were collected and added to HUVECs & the cells were kept at 37°C for 24 hr in 5% CO_2 incubator. HUVECs were viewed under phase contrast microscope and exosomes were viewed under green fluorescent filter in EVOS life imaging system. Finally, the images were merged to confirm the uptake of exosomes by HUVECs.

Treatment of HUVECs with exosomes

HUVECs were treated with pooled RHD patients' serum exosomes (R-exo) and normal individuals' serum exosomes (N-exo) at 1:10 dilution for 48 hr, to evaluate the role of exosomes in HUVECs, after determination of exosomes protein content (1:10=40 µg exosomes) **[16].**

Cell proliferation assay

A rapid cell proliferation assay kit was used to check proliferation of the cells (Biovision, USA). In 96-well plates, HUVECs (2 X 10_4) were seeded in a final volume of 100 µl/well culture medium followed by treatment with N- Exo and R-Exo at 1:10 dilution incubation for 48 h. WST-1/ECS (10 µl/well) solution to each well was added. Cells were incubated for 2 hr in standard culture conditions. Plate was shaken for 1 min on a shaker. The absorbance of N - Exo and R- Exo treated HUVECs was measured by using a microtitre plate reader at 420 nm.

Annexin V/ PI staining

Annexin V – Propidium Iodide staining assay was performed with Annexin V-FITC Apoptosis detection kit (Biovision, USA) in-order to check the apoptosis in HUVECs. The cells were stimulated with N-Exo and R-Exo separately at 1:10 dilution for 48 hours. Further, 5 X 10^5 cells were collected by centrifugation and resuspended in 1X Binding buffer (500 µm µl) followed by addition of Annexin V-FITC (5 µl) and propidium iodide (5 µl). Cells were incubated in the dark at RT for 5 min. Analysis of Annexin V -FITC binding was done by BD FACS Canto II flow cytometer.

Matrigel assay:

Matrigel assay (tube formation assay) was performed using Angiogenesis assay kit (Biovision, USA) in-order to check whether angiogenesis takes place in exosomes treated HUVECs or not. HUVECs were grown up to 90% confluency at 37^0 C incubator containing 5% CO_2. Cells were harvested under sterile conditions and resuspended in desired culture medium containing 5% serum. Thawed extracellular matrix (50 µl) was added to each well of pre-chilled 96 well sterile culture plate and incubated for 1 hr at 37^o C to allow the solution to form the gel. Approximately 1X 10^4 endothelial cells were seeded using 100 µl culture medium. For inhibitor control (suramin) wells, same number of cells were kept in incubator in 100 µl culture medium containing desired concentration of suramin. HUVECs were seeded onto solidified extracellular matrix gel, treated with N- Exo and R-Exo and grown at 1:10 dilution for 48 hours at 37^0 C in CO_2 incubator and the images were captured under EVOS cell imaging system (Thermofischer, USA).

Total RNA extraction

Total exosomal RNA was extracted from biological fluids (serum & PF) and media using miRNeasy mini kit (Quigen, Germany) and total RNA from HUVECs and mitral valve tissue, using Trizol reagent (Invitrogen; Thermofisher, USA) according to manufacturer's instructions. The concentration and quality of RNA were assessed using NanoDrop 2000 Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc). Total RNA from cells and tissues was reverse-transcribed to cDNA immediately for further use **(Table 1).**

Table 1: Primers used in the study

VE cadherin	(F) - CTTCACCCAGAGACCAAGTACACA (R) – AATGGTGAAAGCGTCCTGGT	156
SMA-α	(F) - TATCCCCGGGACTAAGACGC (R) -CACCATCACCCCCTGATGTC	185
TGF-β	(F) - CTCCGAAAATGCCATCCCGC (R) - GCTCAATCCGTTGTTCAGGC	164
Furin	(F) - CCCTCAACCTCCTCTTCT (R) - CACCAACCCAGCATCTTAC	198
VEGF	(F) - TCACCATGCAGATTATGCGGA (R) - CAACGTACACGCTCCAGGAC	198
U6	(F)- GCTTCGGCAGCACATATACTAAAAT (R) CGCTTCACGAATTTGCGTGTCAT	91

miRNA sequencing

Preparation of library and sequencing of small RNA were carried out by using exosomal RNA isolated from serum, PF and stimulated HUVECs media (RHD *in-vitro* model), using an Illumina HiSeq™ 2500 device (Biologia Research India Pvt. Ltd.) Concatenation of whole exosomal RNA with 5'- and 3' adaptors was performed. Following cDNA synthesis and PCR amplification, an acrylamide gel purification procedure was used to prepare the cDNA library (18-40 nt), which was then sequenced.

RT-qPCR validation for selected miRNA

A common exosomal miRNA (miR-24-2) from serum, PF and stimulated HUVECs media (RHD *in-vitro* model) was selected via miRNA profiling with (Cq>=2), and, qRT-PCR for specific miRNA-24-2 was performed in exosomes isolated from biological samples, N exo & R-exo treated HUVECs and mitral valve tissues. RNA was reversed-transcribed and the expression level of miRNA-24-2 was measured using miRVana miRNA qRTCR detection kit following the manufacturer's instructions. The primers were available in the kit. For internal control, U6 was used to normalize the relative expression ratio of miRNA. The $2^{-\Delta\Delta Cq}$ method were used to calculate fold change (Roche, Germany). The PCR conditions were as follows: holding stage: 95˚C for180 sec; cycling stage: 95˚C for 15 seconds and 60˚C for 30 sec, 40 cycles **(Table 1).**

Target gene prediction:

Furin was the predicted target gene of miR-24-2, which was analysed by extensive literature search and using miRDB software **[17].** HUVECs were transfected with miRs and anti-miRs specific for the selected miRNA-24-2. Next, miRNA and target gene (furin) expression was evaluated. Furin, enhances the levels of downstream TGF-β during cardiac fibrosis via the furin-TGF-β pathway **[18].** Furin expression levels were estimated in exo-treated HUVECs and mitral valve tissue of RHD patients. Further, downstream TGF-β gene expression levels (fibrotic gene, indicative of fibrosis) were also evaluated in the mitral valve tissues of RHD patients. Total RNA was reverse transcribed and measured by using SYBR I green (Thermofischer, USA). GAPDH was used as an internal control to normalize the relative expression of RNA, using the $2^{-\Delta\Delta Cq}$ method in Light Cycler Roche 96 PCR system (Roche, Germany) for RT-PCR analysis.

Transfection of exosomes treated HUVECs with miR-24-2 specific miRs and anti-miRs

miRVana miRNA-24-2 specific miRs and antimiRs and scrabled miRNA (negetive control) was purchased from Thermofischer Scientific, USA. Transfection was performed using Lipofectamine RNAi MAX (Thermofisher Scientific, USA) according to manufacturer's protocol. Briefly, lipofectamine RNAi MAX reagent (3 µl) was diluted in 50 µl endothelial cell growth media (Sigma Aldrich, USA), without antibiotics and FBS. Simultaneously, 25 nM of miRs/antimiRs (siRNA) was diluted in same media, without antibiotics. Lipofectamine RNAiMAX reagent and siRNA was mixed in 1:1 ratio and incubated for 5 min at RT. For forward and reverse transfection, $2X10^5$ cells were seeded in 12 well plate to get 60-80% confluency and 50 µl of above mixture was added to wells. HUVECs were cultured in 5 settings; HUVECs treated with R-exo, HUVECs treated with R-exo-transfected with miR-24-2 specific miRs, HUVECs treated with N-exo and HUVECs treated with N-exo- transfected with miR-24-2 specific anti-miRs and scrambled miRs at 1:10 dilution for 48 h. The morphology of these cells were observed, followed by trypsinisation and RNA isolation. The gene expression analysis for selected miRNA-24-2 and its target gene, furin, was also carried out. EndoMT markers (VE cadherin and SMA-α) and fibrotic genes (TGF-β) gene expression levels was also evaluated.

Haematoxylin-Eosin staining of mitral valves tissues

To check for mitral valves fibrosis, Haematoxylin-Eosin staining was performed using standardised protocol. The slides with section were placed in a metal staining rack. The

sections were immersed in the filtered Harris Hematoxylin for 10 sec. The tap water was exchanged until the water is clear. The sections were immersed in EOSIN stain for 30 sec. The washing step was repeated. The sections were dehydrated in ascending alcohol solutions (50%,70%,80%,95%,100%) in Columbia staining dish. The sections were then cleared with xylene in Columbia staining dish. The coverslip was mounted onto the section on glass slide with suitable organic mounting medium and the sections were viewed under the microscope.

Mason trichrome staining of mitral valves tissues

To confirm, mitral valves fibrosis, fibrosis specific stains, mason trichrome staining was also performed using standardised protocol. Weigert's iron hematoxylin stains the nuclei in black, Biebrich scarlet-acid fuchsin stains cytoplasm & muscle fibers in red and after treatment with phosphotungstic and phosphomolybdic acid, collagen is stained in blue with aniline blue. To prevent the incubation solutions from running away, the slides were warmed to RT and portions were wrapped with a liquid blocker. The cryosections were preserved in 4 % PFA or 10% formalin for 1 hr in the hood at 37° C. To heighten the colours and increase the contrast between the tissue components, sections were re-fixed in Bouin's solution overnight. Slides were rinsed in deionized/distilled water after being cleaned for 1-2 min under running tap water (18–26°C) to remove yellow colour from sections. To stain the nuclei dark, equal volumes of Hematoxylin Solution A and Solution B were mixed and the sections were treated for 5 min with Weigert's Iron Hematoxylin Solution. Hematoxilin solution was discarded. The slides were placed in a glass chamber and washed for 10 min in warm flowing tap water to remove excess hematoxylin and deepen the black colour, followed by 1 min rinse in water. To stain the fibers red, the sections were treated in Biebrich Scarlet-Acid Fuchsin Solution for 5 min. Finally, the solution was discarded, and the tissues were examined under a microscope.

Statistical analysis: The study was done in triplicate and the results were expressed as the mean SD. Student's t-test was used to compare data between two groups, and one-way analysis of variance (ANOVA) was used to analyse data from more than two groups. A p-value of 0.05 was considered statistically significant.

3. Results

Human umbilical cord vein endothelial cells (HUVECs) culture: HUVECs were cultured in endothelial cell growth medium (Sigma Aldrich, USA) and maintained up-to 4 days. The cultured HUVECs showed cobblestone morphology (Figure 1A) with oval nuclei and irregular cell shape throughout the experiment. The isolated HUVECs were confirmed by indirect immuno-fluorescence, using antibody against endothelial cell surface marker CD-31 (Figure 1B) and proceeded for further analysis.

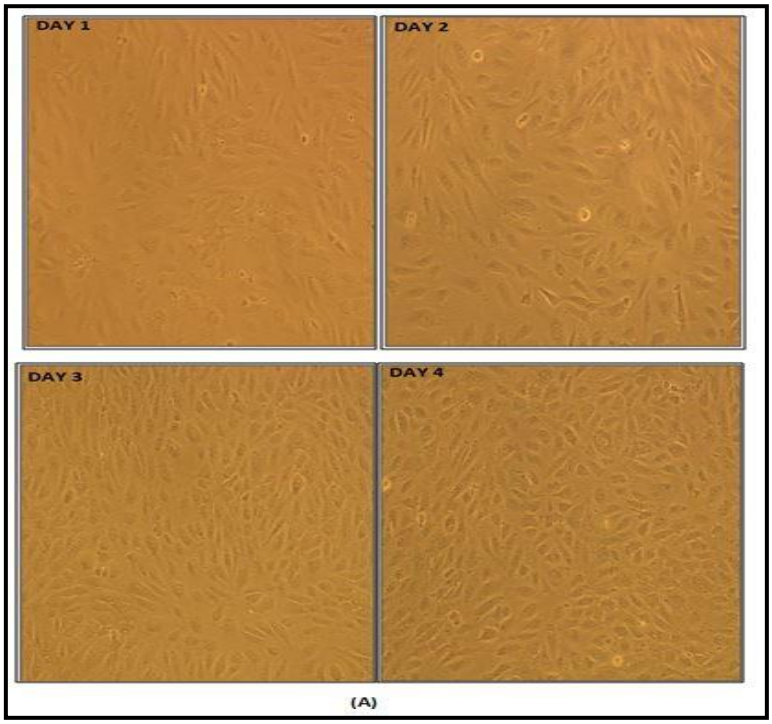


Figure 1(A):Human umbilical cord vein endothelial cells (HUVECs) culture and maintenance: The isolated HUVECs were cultured and maintained in endothelial cell growth medium upto 4 days. Rounded cobblestone morphology of HUVECs were observed 10(X) Scale bar = 400 µm.

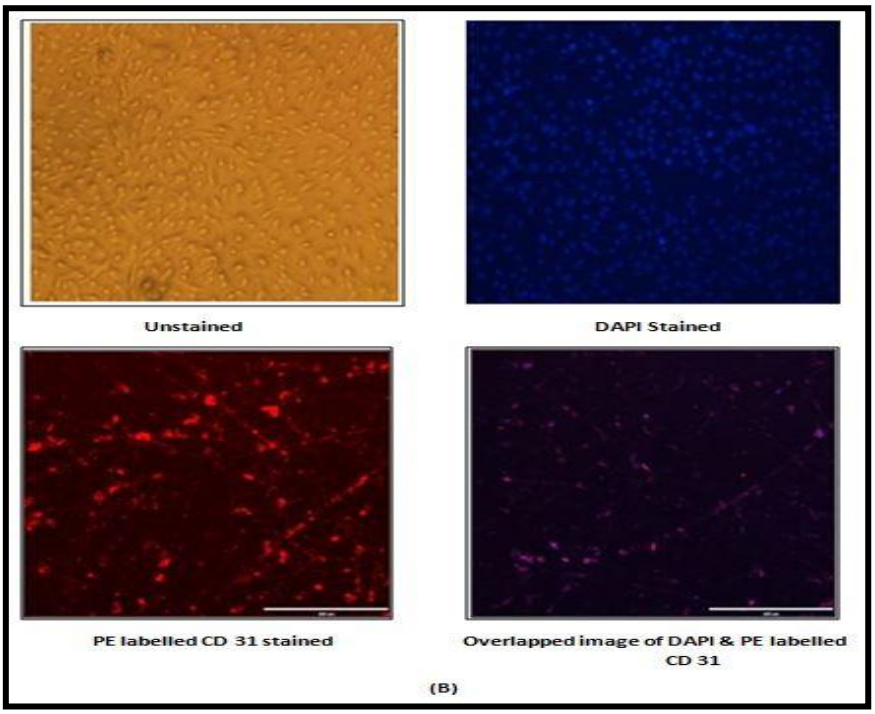
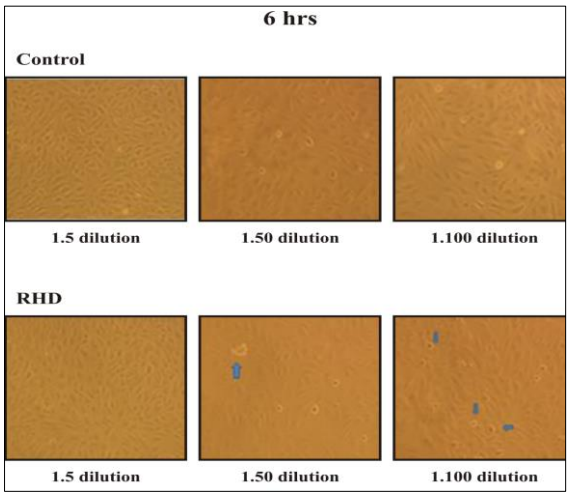


Figure 1(B):Human umbilical cord vein endothelial cells (HUVECs) culture and maintenance: The isolated HUVECs were cultured and maintained in endothelial cell growth medium upto 4 days. Rounded cobblestone morphology of HUVECs were observed 10(X) Scale bar = 400 µm.

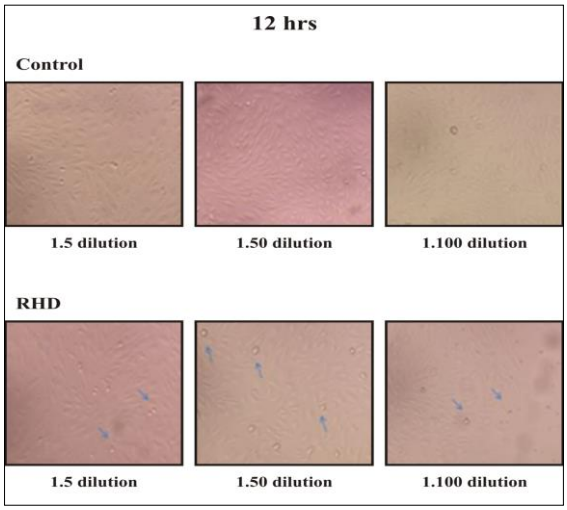
Stimulation of HUVECs with RHD patients' pooled serum

In-order to develop *in-vitro* RHD model, HUVECs (primary cells) were stimulated by pooled RHD serum at 3 different dilutions 1:5, 1:50 and 1:100 at different time intervals of 6h and 12h. HUVECs showed a rounded appearance at 1:50 and 1:100 dilutions at 6 and 12 hours more as compared to cells stimulated with pooled RHD patients' serum at 1:5 dilution for 6 and 12 hours (Figure 2A). However, no such morphological difference was observed in HUVECs treated with healthy controls' serum.

Further, to confirm whether HUVECs were stimulated with pooled serum from RHD patients, the expression levels of several genes : TNF-α, ($P<0.01$) INF-γ ($P<0.01$), vCAM ($P<0.001$), IL-1β($P<0.001$) involved in RHD pathogenesis **[14]** was estimated and all these genes showed significant augmentation, correlating with the development of *in -vitro* RHD model (Figure 2B).



(A-1)



(A-2)

Figure 2(A). Stimulation of HUVECs with pooled RHD patients' serum: Representative micrograph of HUVECs showing rounded bodies (arrow) upon stimulation with RHD patients' pooled serum at 3 different dilutions for 6 **(A-1)** and 12 hours **(A-2)** (10X). Scale bar = 400 µm. Arrows show the change in morphology of HUVECs

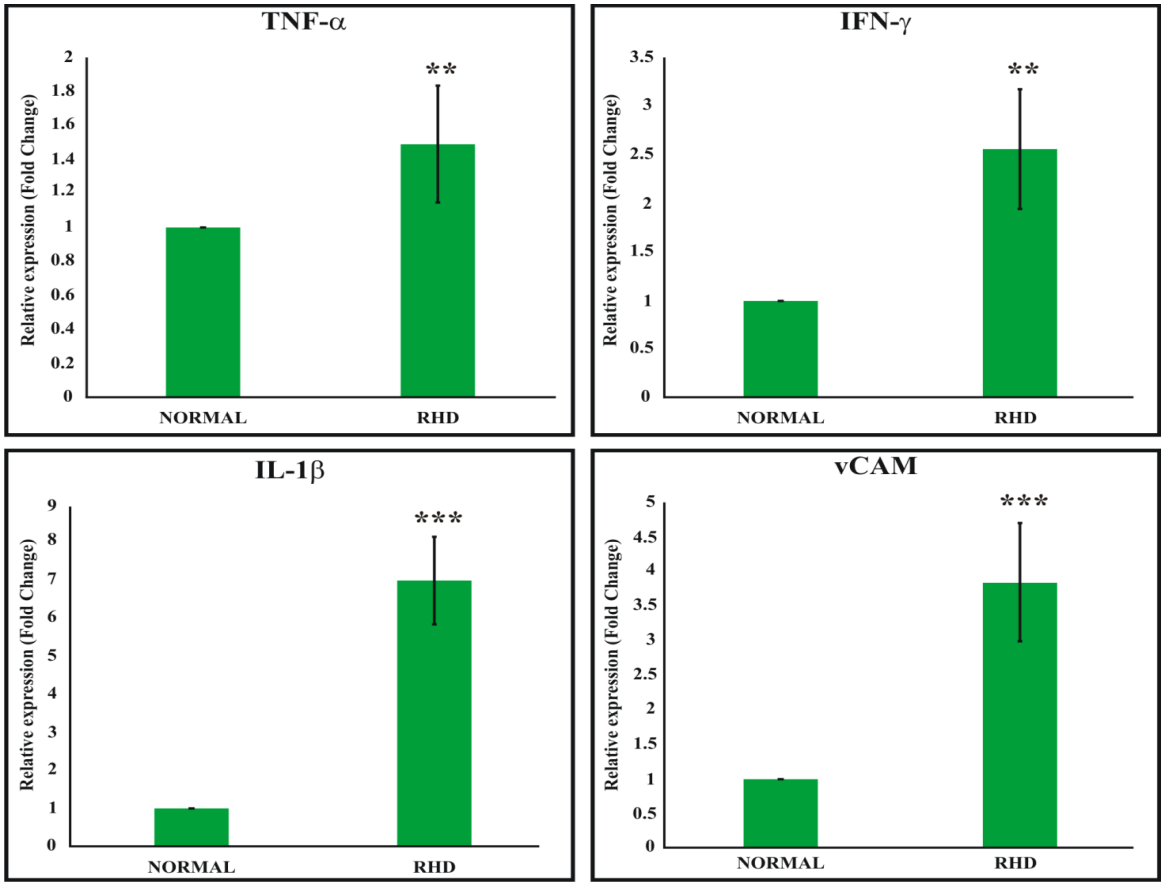


Figure 2 (B) Relative gene expression of various pro-inflammatory genes associated with RHD: (a) TNF-α, (b) INF-γ, (c) IL-1β (d) vCAM, in pooled RHD patients' serum stimulated HUVECs (RHD *in-vitro* model) as compared to control (HUVECs without stimulation). Experiments were performed in triplicate and data is represented as mean ± SD. Statistical significance was calculated by Student's t-tests. **p < 0.01, ***p < 0.001.

Isolation of exosomes

Exosomes were isolated from RHD patients' serum and PF samples. Exosomes were also isolated from stimulated HUVECs media (RHD *in-vitro* model) and their respective controls. The isolated exosomes were characterised using TEM, Zeta sizer and western blot. Transmission electron microscopy images confirmed that the isolated vesicles as exosomes with size range of 10-150 nm and typical cup-shaped morphology. The exosomes were further characterized by using Zeta Sizer to evaluate the percentage intensity of vesicles in different size range. Zeta sizing of isolated exosomes showed that 90% of vesicles lie in the size range of exosomes, with a diameter of 30.89 nm, 34.69 nm (RHD patients' serum and controls serum), 37.4 nm, 136.1 nm (RHD patients' PF and CABG patients' PF), 121.3 nm and 24.2 nm stimulated HUVECs media (RHD *in-vitro* model) and HUVECs without stimulation, respectively, as the size of the vesicles examined was same as the size range of exosomes (10-150 nm), thereby, indicating that the isolated vesicles were exosomes. Further, western blot analysis of isolated exosomes using surface marker protein CD-63 was performed and a 65 KDa protein band specific to exosomal surface marker CD 63 was identified **(Figure 3): published data .**

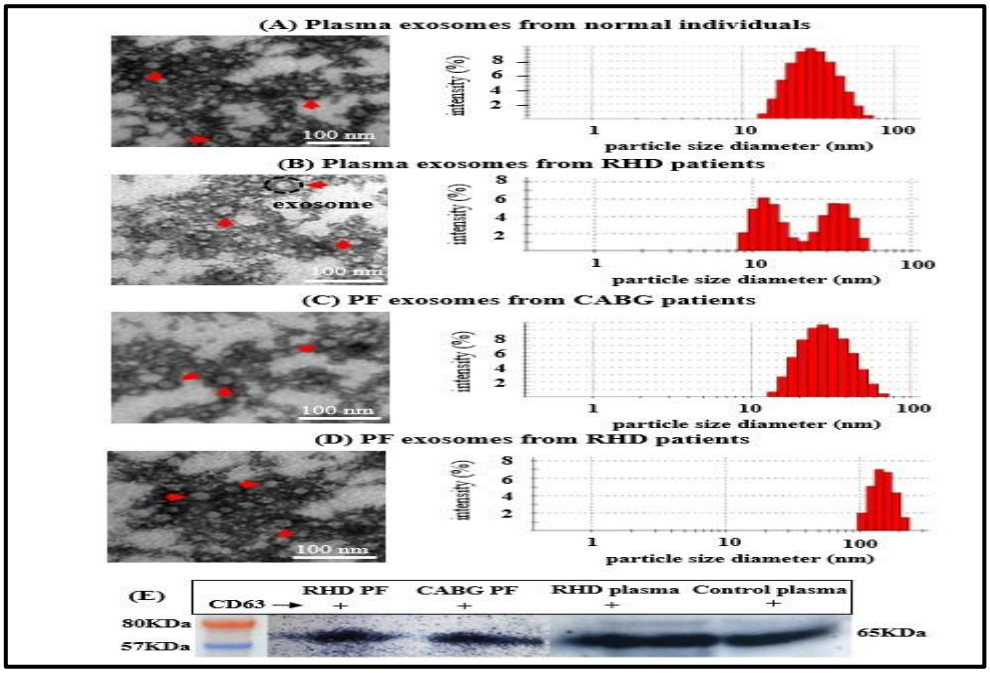
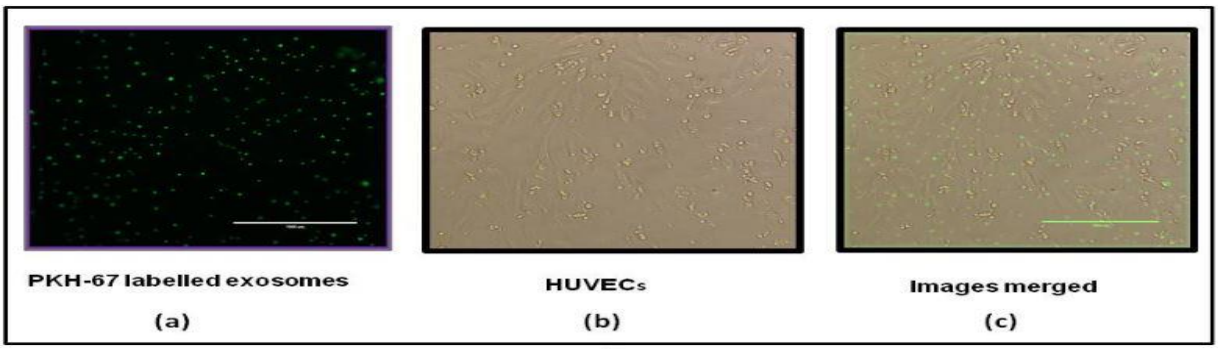


Figure 3. Characterization of exosomes from biological fluids from RHD patients and controls by TEM, Zeta Sizer (A-D) and Western Blot (E) : published data

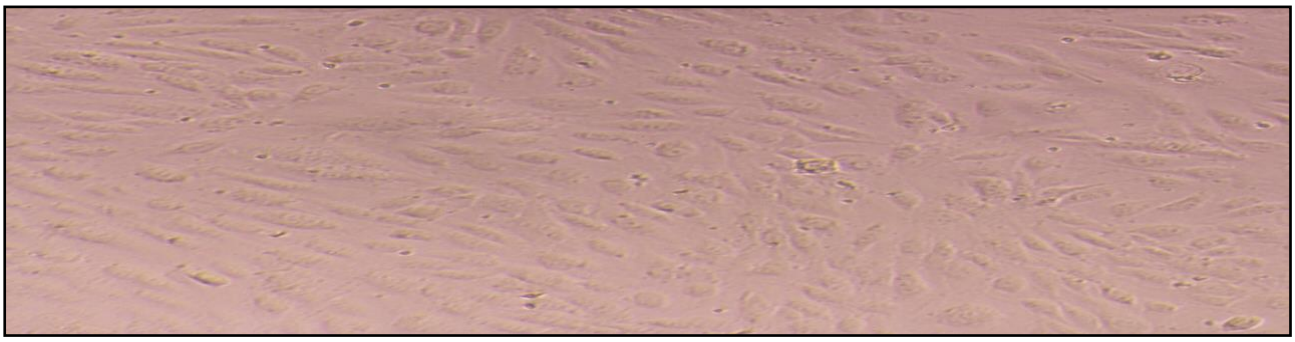
Effect of exosomes treatment *in- vitro*

PKH-67, labels lipophilic membrane of the exosomes. Initially, PKH-67 labelled exosomes were collected and added to HUVECs & the cells were kept at 37°C for 24 hours in 5% CO_2 incubator. Exosomes were viewed under green fluorescent filter and HUVECs were observed under phase contrast microscope in EVOS life imaging system, and images were merged which confirmed the uptake of exosomes by HUVECs (Figure 4A). HUVECs were further, treated with exosomes isolated from RHD patient's serum exosomes(R-exo) and normal individuals' serum exosomes (N-exo) to check whether stimulation with exosomes brings about any morphological changes at 1:10 dilution for 48 h and it was observed that there was a distinct morphological change in N-exo treated-HUVECs, at 1:10 dilution for 48 hours. However, rounded bodies could be seen in R-exo treated-HUVECs, indicative of morphological changes associated with treatment of HUVECs with exosomes (Figure 4B).

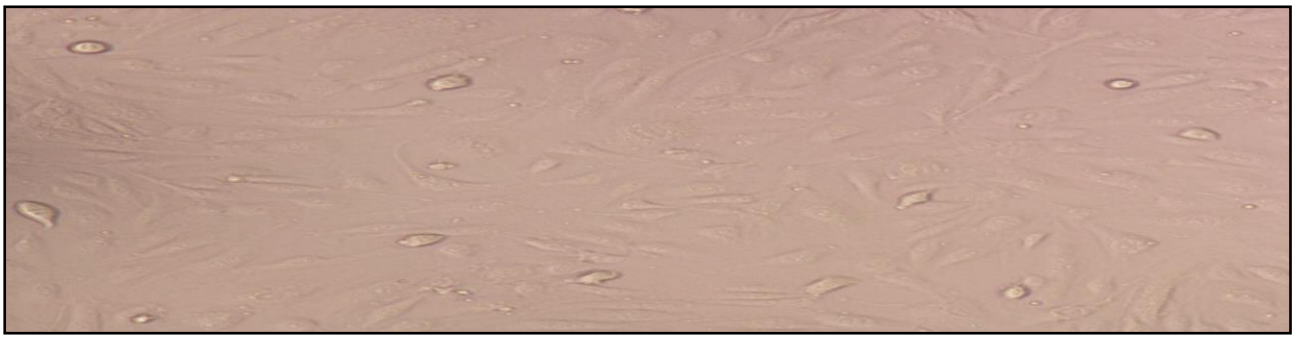


(A)

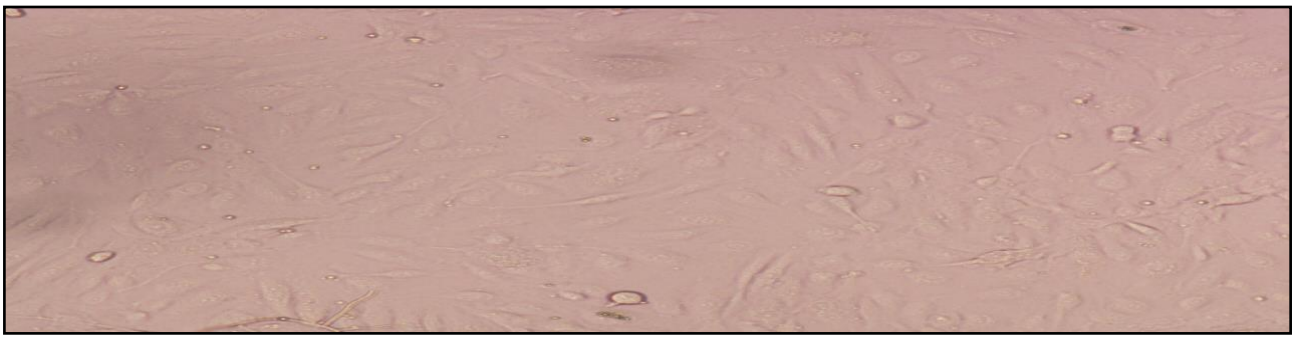
Figure 4(A) Uptake of PKH-67 labelled exosomes by HUVECs: Representative images of (a) PKH-67-labelled exosomes, (b) HUVECs and (c) overlapped images showing exosomes uptake by HUVECs, 10(X). Scale bar = 400 µm



(a)PBS treated HUVECs



(b) R-exo treated HUVECs



(c) N-exo treated HUVECs

(B)

Figure 4(B) Effect of treatment of HUVECs with exosomes, isolated from RHD patients: Representative micrograph of HUVECs treated by (a) PBS, (b) exosomes isolated from RHD patients' serum (R-exo) and (c) normal individuals serum (N-exo) at 1:10 at 48 h, (10X) Scale bar = 400 µm

Next, changes in biological properties such as cell, proliferation, apoptosis, angiogenesis and expression levels of EndoMT markers were evaluated in exosomes treated HUVECs. The cell proliferation assay was conducted using cell proliferation assay kit (Biovision, USA), and it was observed that R-exo treated-HUVECs significantly decreased ($p<0.01$) in cell number as compared to N-exo treated HUVECs (Figure 5a). It was interesting to check, whether this reduced cell number is due to increased apoptosis, Annexin V/PI staining of these cells was performed using Annexin V/PI assay kit (Biovision, USA), which revealed an overall significant increase in the percentage of apoptotic cell in R-exo treated-HUVECs ($p<0.01$) as compared to N-exo treated-HUVECs (Figure 5b & c).

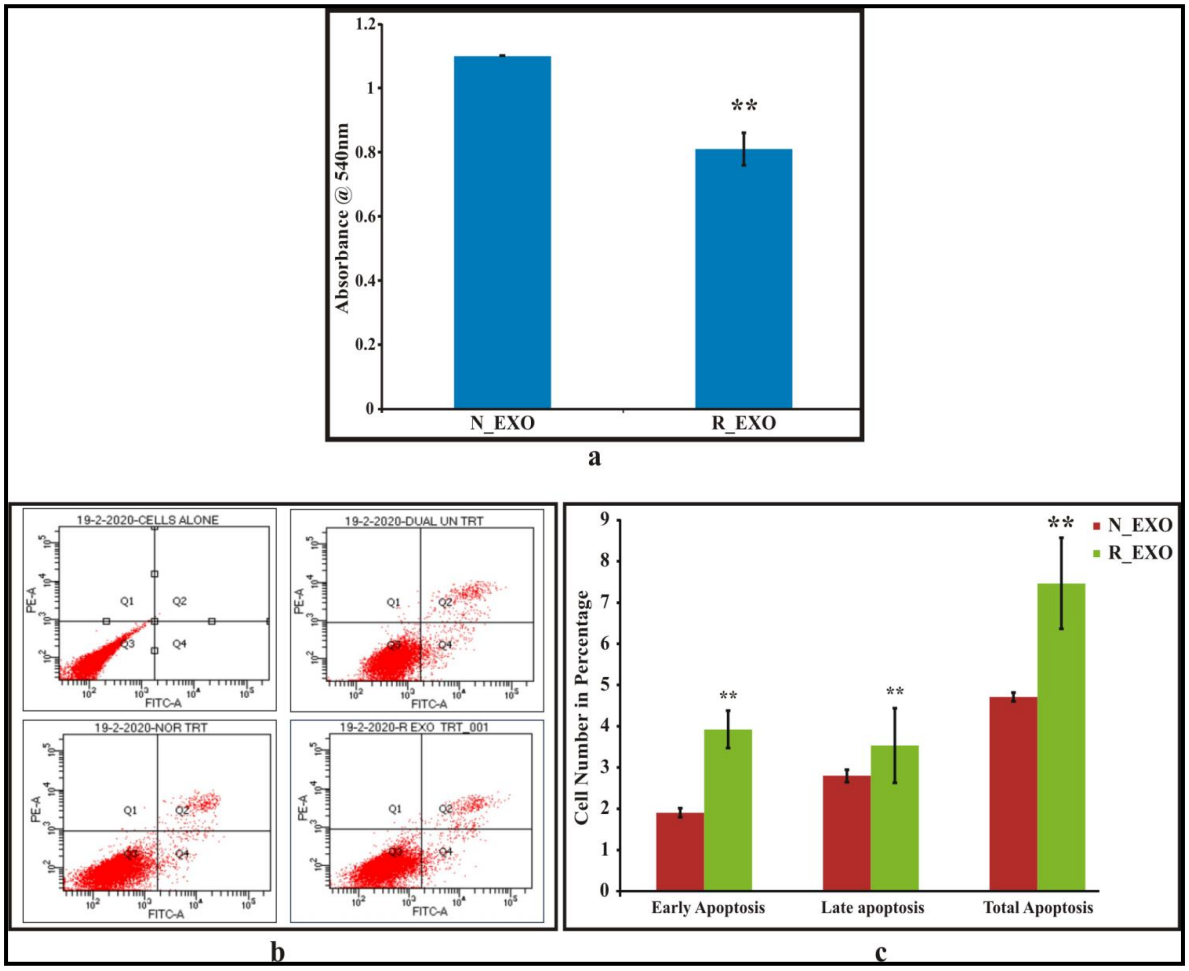


Figure 5. Effect of treatment of exosomes on proliferation and apoptosis in HUVECs: **(**a) Cell proliferation assay showing relative cell number. (b) the percentage of apoptotic cells were measured by flow cytometry using the annexin V-PI assay (Nor Trt= N-exo treated, R trt=R-exo treated, DU trt= dual untreated) (c) Bar diagram showing the percentage of cells undergoing apoptosis. All experiments were repeated three times. Each bar represents the mean ± S.D. Statistical analysis was carried out by student's t- test. **P < 0.01 compared to control.

Next, matrigel angiogenesis assay was performed in N-exo and R-exo treated HUVECs, using tube formation assay kit specific for angiogenesis (Biovision, USA) and the images showed the beginning of angiogenesis in N-exo treated- HUVECs, whereas no angiogenesis was observed in R-exo treated HUVECs (Figure 6). Next, to confirm these observations that angiogenesis occurs in N-exo treated-HUVECs and apoptosis occurs in R-exo treated-HUVECs, gene expression of VEGF (angiogenic gene) and caspase-3 (apoptotic gene) in exosomes-treated HUVECs was estimated. It was found that the expression levels of Caspase-3 significantly augmented in R-exo treated HUVECs ($P < 0.001$), as compared to N-exo treated-HUVECs. However, the expression of VEG-F was found to be significantly attenuated in R-exo treated-HUVECs ($P < 0.001$) as compared to N-exo treated HUVECs (Figure 7a). These results, thereby, confirmed the changes in morphology and biological properties in exosomes-treated HUVECs.

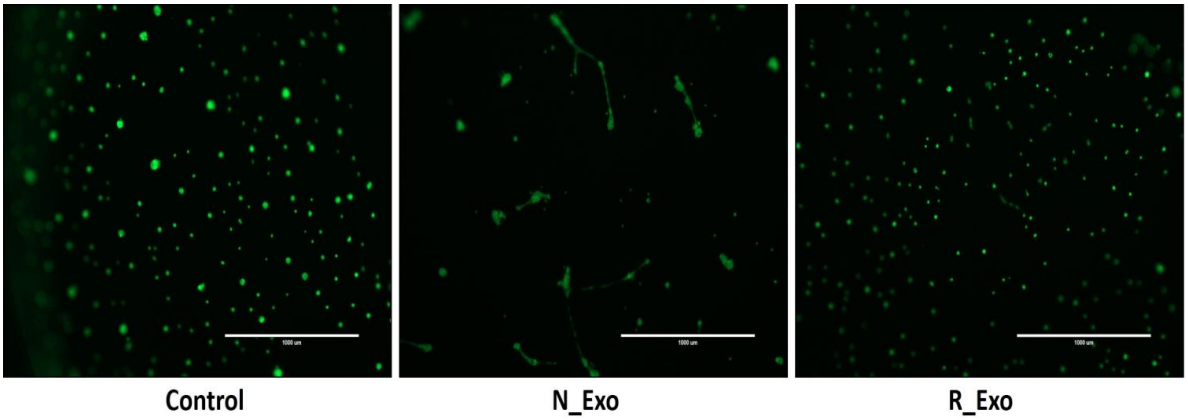


Figure 6: Effect of treatment of exosomes on angiogenesis in HUVECs: Representative micrograph of the matrigel angiogenesis assay in normal individuals exosomes (N-exo) treated HUVECs; RHD patients exosomes (R-exo) treated HUVECs as compared to suramin control(angiogenesis inhibitor), (10X). Scale bar = 400 µm

Gene expression levels of mesenchymal markers and endothelial markers in exosomes treated HUVECs

Mitral valve fibrosis is caused by increased TGF-β production, by myofibroblasts (mesenchymal in origin), therefore, the expression levels of mesenchymal markers (SMA-α) and endothelial cell markers (VE-cadherin) in N-exo treated HUVECs and R-exo treated-HUVECs was estimated. It was found that the levels of SMA-α significantly enhanced ($P < 0.001$) and levels of VE-cadherin significantly declined in R-exo treated-HUVECs ($P < 0.001$) as compared to N-exo treated-HUVECs (Figure 7b).

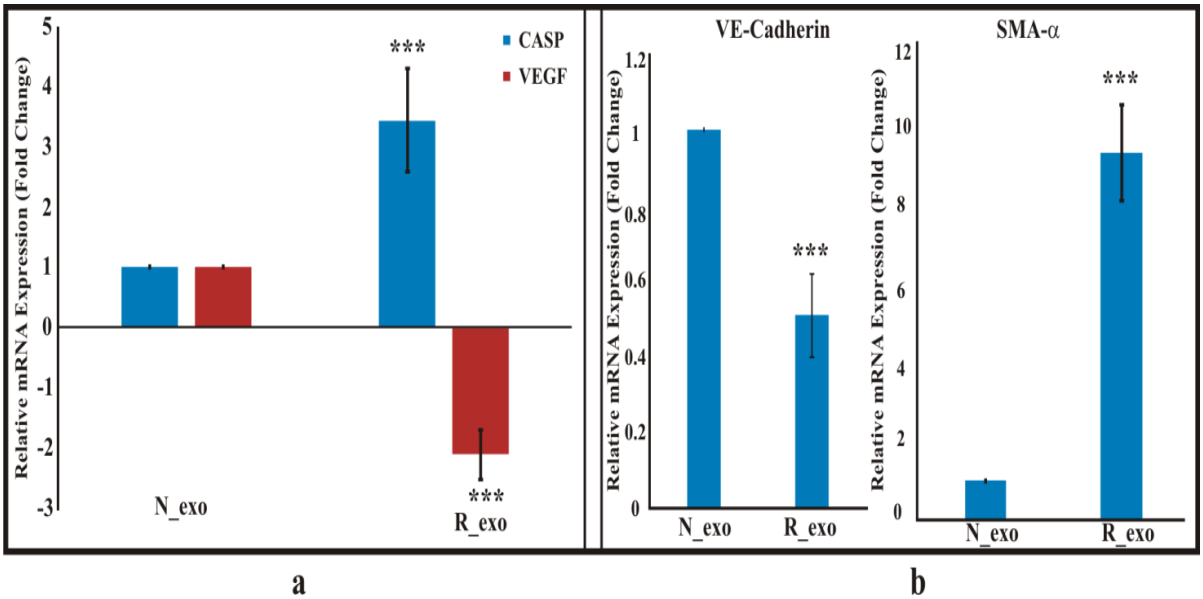
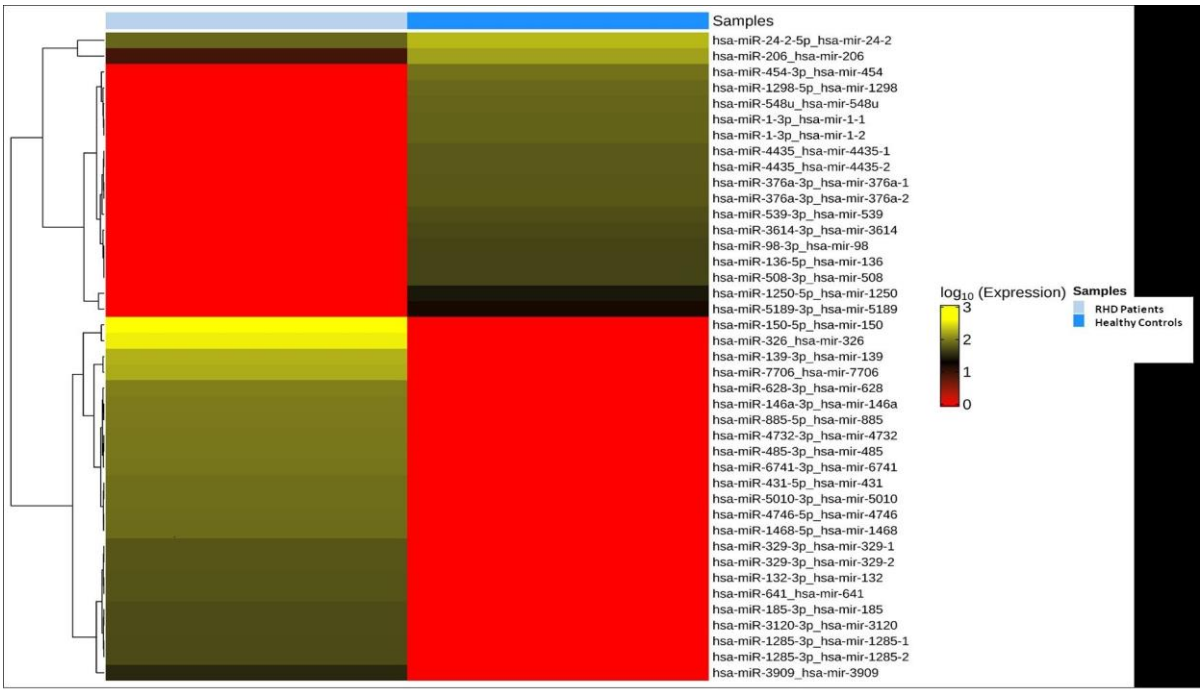


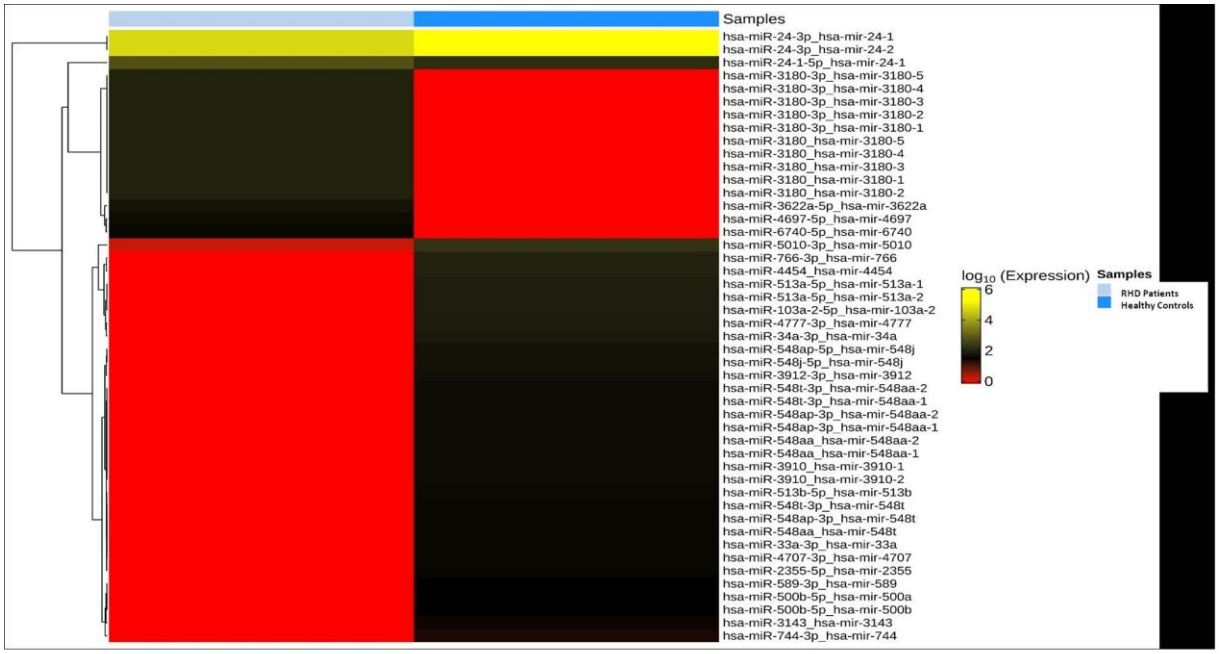
Figure 7: Gene expression analysis of angiogenic, apoptotic and EndoMT genes in exosomes treated HUVECs (a) RT-PCR of angiogenic gene (VEG-F) and apoptotic gene (caspase-3) (b) endothelial (VE-cadherin) and mesenchymal markers (SMA-α).All experiments were repeated three times. Each bar represents the mean ± S.D. Statistical analysis was carried out by student's t- test. ***P < 0.001 compared to control.

Selection of exosomal miR-24-2

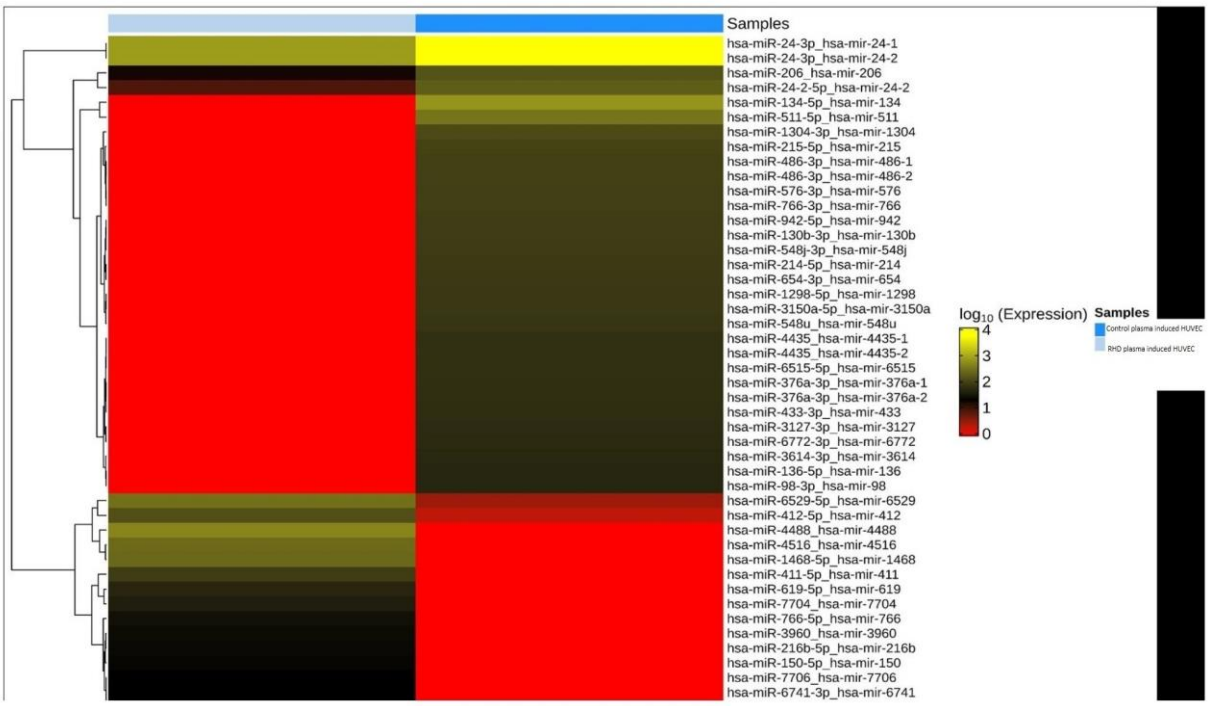
Initially, exosomes were isolated from serum and PF of RHD patients and stimulted HUVECs media (RHD *in-vitro* model), including their respective controls. RNA was isolated from these exosomes and subjected to miRNA expression profiling, which was outsourced from Biologia Research India Pvt Ltd. Briefly, all samples were mapped to Human reference genome (GRCh38.p13) using Bowtie (v1.3.0). Mirdeep2 (v0.1.2) is used to discover, identification and expression of miRNAs based on latest miRBase release (v22). Differential expression analysis has been done using edgeR (3.28.1). All heatmaps has been generated from log (row count data) by using Complex Heatmap (version: 2.2.0). The Gene chips represents different fold changes from many up-regulated miRNAs and down-regulated miRNAs by heat map of microRNA expression data (Supplementary Figure 1-3). A total of 2653 miRNAs were detected in overall. Further miRNAs with ΔΔ Cq valves>=2 was selected as a criteria for selection of de-regulated miRNAs **[19],** where exosomal miR-24-2, was screened as a common miRNA with down-regulated expression in patient samples (*in vivo samples*) and media (*in-vitro*). As, studies, suggested the role of miR-24-2 in cardiac EndoMT and fibrosis also **[12,13],** therefore, exosomal miR-24-2 was selected for further analysis.



Supplementary Figure 1: Exosomal miRNA profiling in pooled RHD patients' serum showing differentially expressed miRNAs; yellow, upregulated miRNAs; grey, not differentially expressed miRNA; red, downregulated miRNAs. The criteria required a minimum of twofold difference of log2 (fold change) in either direction.



Supplementary Figure 2: Exosomal miRNA profiling in pooled RHD patients' PF, showing differentially expressed miRNAs; yellow, upregulated miRNAs; grey, not differentially expressed miRNA; red, downregulated miRNAs. The criteria required a minimum of twofold difference of log2 (fold change) in either direction



Supplementary Figure 3: Exosomal miRNA profiling in HUVECs stimulated media (RHD *in-vitro* model) showing differentially expressed miRNAs; yellow, upregulated miRNAs; grey, not differentially expressed miRNA; red, downregulated miRNAs. The criteria required a minimum of twofold difference of log2 (fold change) in either direction.

miR-24-2 expression levels and its target gene prediction:

Exosomal miRNA-profiling was outsourced to screen out de-regulated miRNAs, where, the potent exosomal miR-24-2, (ΔΔ Cq>2) was selected as a common miRNA in all the samples (serum, PF and media) involved in EndoMT and cardiac fibrosis. Therefore, the expression levels of miR-24-2 were checked in serum exosomes, PF exosomes, mitral valves tissues of RHD patients and R-exo and N-exo-treated HUVECs. Total 90 samples were used (serum, PF samples, mitral valve tissues) to evaluate the expression levels of miR-24-2. It was observed that the expression levels of miR-24-2 was significantly reduced in all RHD patients' samples (serum, PF, mitral valves) (P<0.001) including R-exo-treated HUVECs as compared to their respective controls (Figure 8).

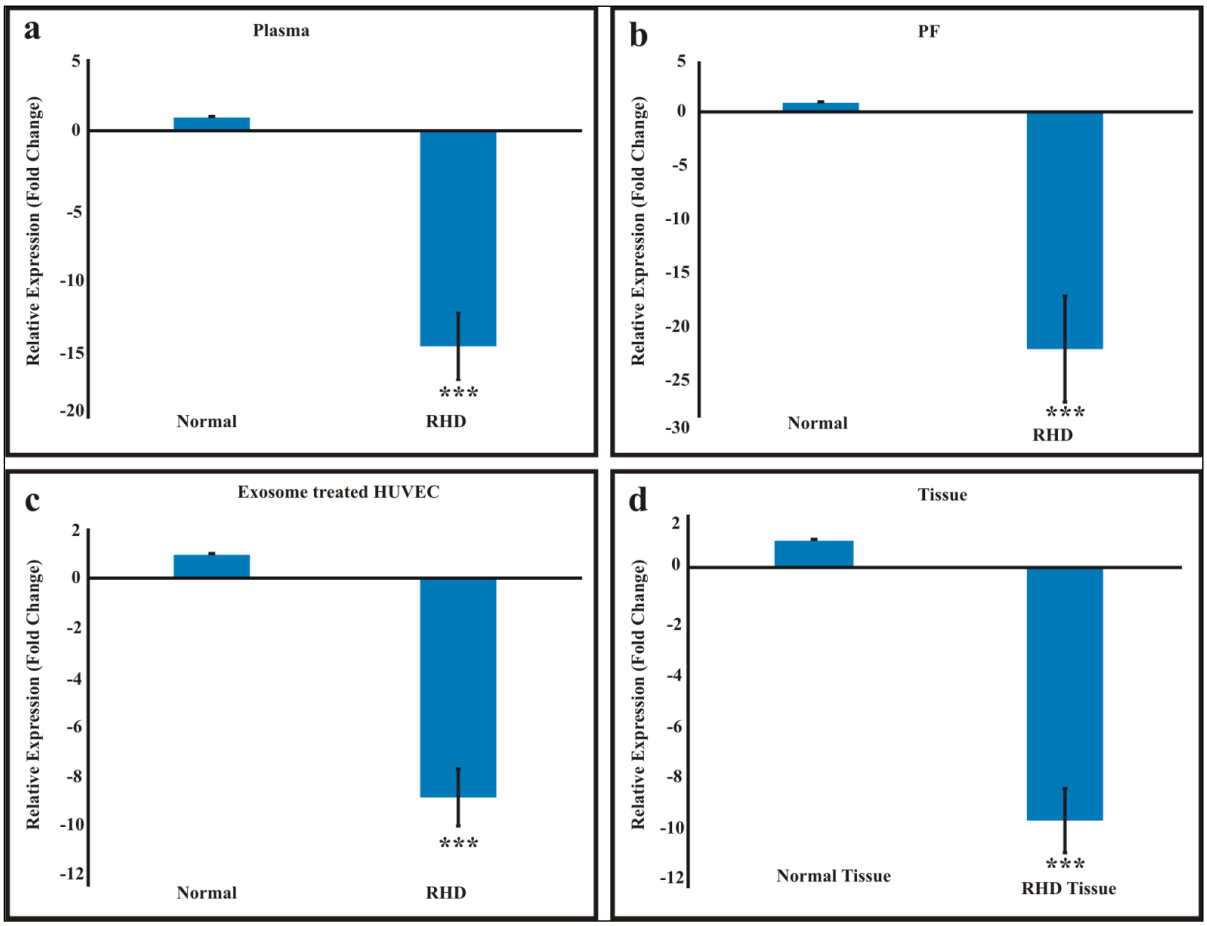
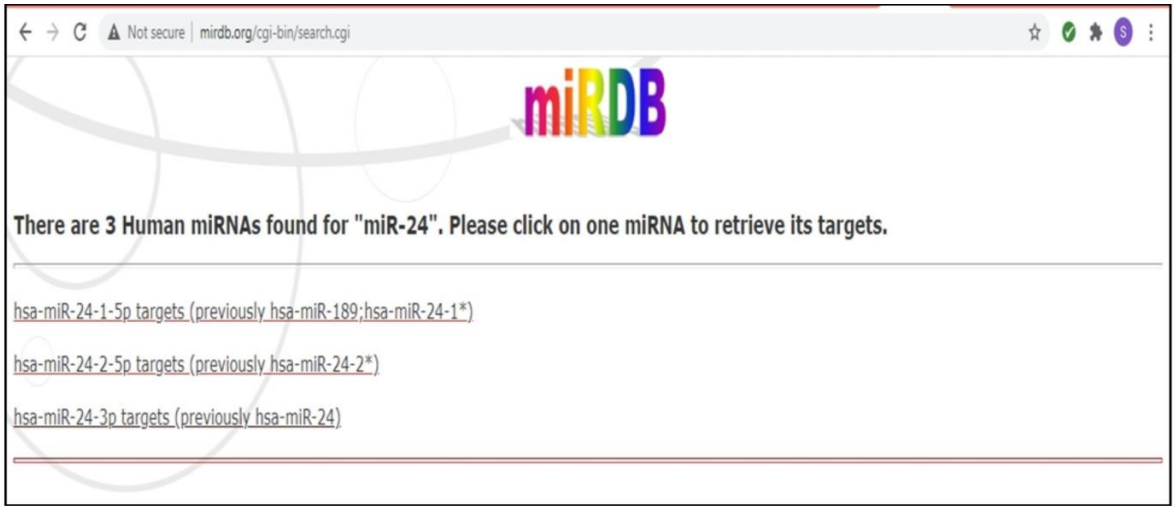
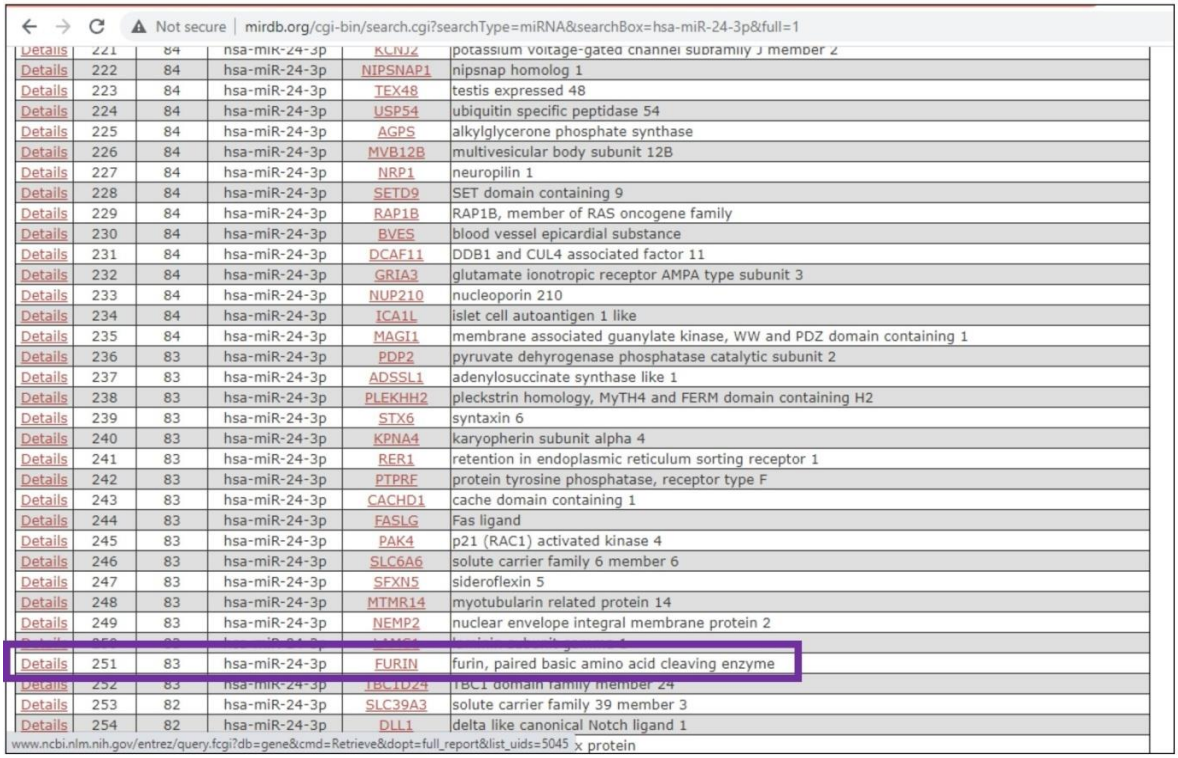


Figure 8: miR-24 (miR-24-2) expression levels in the samples: RT-PCR of miR-24-2 in (a) RHD patients' serum samples, (b) RHD patients' PF samples (c) R-exo-treated HUVECs and (d) RHD patients' mitral valve tissues as compared to respective controls. All experiments were repeated three times. Each bar represents the mean ± S.D. Statistical analysis was carried out by student's t- test. ***P < 0.001 compared to control

Further, the target gene for miR-24-2 was evaluated using target gene prediction miRDB software. Literature search and miRDB software analysis revealed that the predicted target gene for miR-24-2 is furin (supplementary fig 4).





Supplementary Figure 4: miR-24-2 target gene prediction using miRDB software analysis: A screenshot for miRDB target gene prediction analysis. Furin is one of the target gene encircled with square

Next, HUVECs were transfected with miR-24-2 miRs/ anti-miRs and furin gene expression was evaluated in transfected HUVECs in comparison to HUVECs without stimulation. It was observed, miR-24-2-miRs transfected HUVECs, showed increased expression levels of miR-24-2 ($P<0.001$) and decreased levels of furin ($P<0.001$), however, opposite trend was observed in HUVECs transfected with miR-24-2 specific anti-miRs, thereby suggesting that miR-24-2 targets furin (Figure 9). Furin is a protease which controls latent TGF-β activation process. Therefore, the expression levels of furin and TGF-β (downstream gene, a pathological mediator that causes cardiac fibrosis) was estimated in R-exo and N-exo treated HUVECs and mitral valves (n=42) of RHD patients. It was found that the gene expression

levels of furin was significantly elevated in R-exo treated HUVECs (P<0.01) as compared to N-exo treated-HUVECs and also the expression levels were significantly elevated in mitral valve tissues of RHD patients (P<0.01) as compared to control tissues. Similarly, the expression levels of TGF-β, significantly augmented (P<0.01) and expression levels of miR-24-2 were significantly reduced (P<0.001) in the mitral valves of RHD tissues as compared to control tissues (Figure10 a,b).

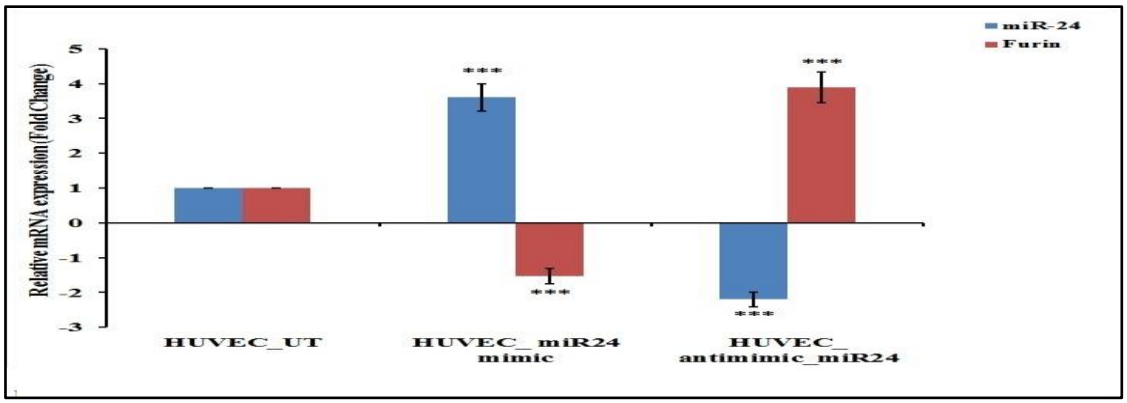


Figure 9: miR-24-2 target gene prediction by gene expression analysis : RT PCR of miR-24-2 and furin, after transfection of HUVECs with miR-24-2 miRs and anti-miRs. All experiments were repeated three times. Each bar represents the mean ± S.D. Statistical analysis was carried out by student's t- test. **P < 0.001 compared to control.

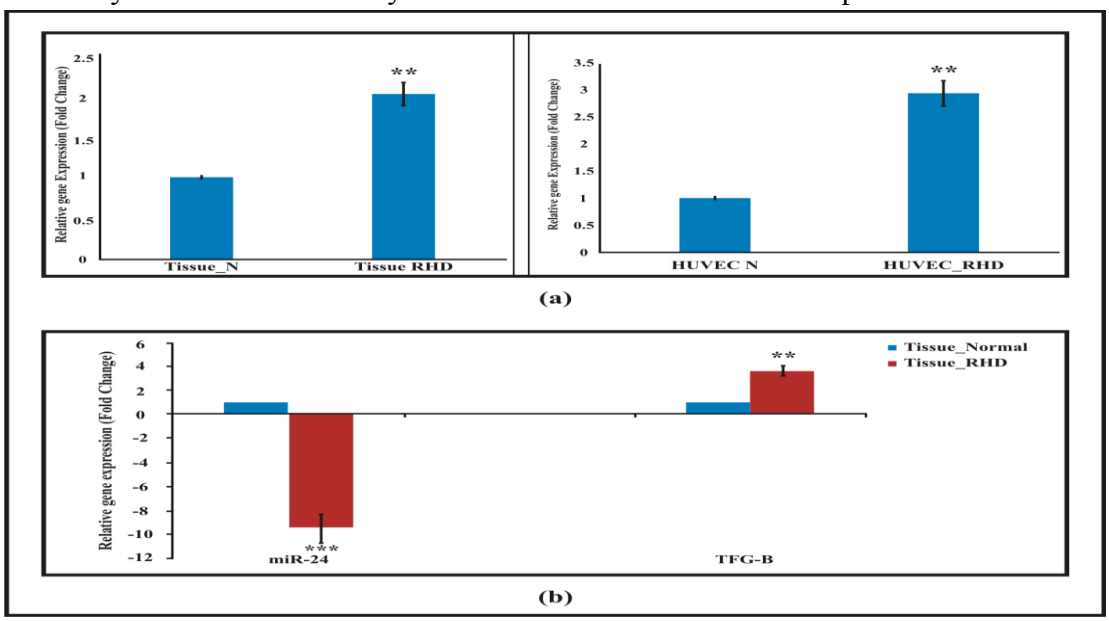


Figure 10(a): Gene expression levels of exosomal miRNA-24-2 target gene (furin) : RT PCR of furin in tissues (Tissues_RHD) and normals (Tissue_N) and R-exo-treated HUVECs (HUVECs_RHD) as compared to N-exo-treated HUVECs (HUVECs_N) **(b) Gene expression levels of exosomal miRNA-24-2 and downstream TGF-β:** RT-PCR of miR-24-2 and TGF-β in mitral valve tissues from RHD patient as compared to control tissues. All experiments were repeated three times. Each bar represents the mean ± S.D. Statistical analysis was carried out by student's t- test. **P < 0.01, ***P < 0.001 compared to control.

Functional inhibition (using miRs and anti-miRs) of exosomal miR-24-2

To study the cause and effect of the study, miRNA functional inhibition was performed in HUVECs using miRs and anti-miRs specific for miR-24-2. The morphological changes was analysed in five settings in HUVECs: HUVECs treated with R-exo, HUVECs treated with R-exo-transfected with miR-24-2 miRs, HUVECs treated with N-exo, HUVECs treated with N-exo-transfected with miR-24-2 anti-miRs and scrambled miRs at 1:10 dilution for 48 h. Interestingly, distorted morphology in HUVECs could be seen in R-exo treated HUVECs as it has been observed R-exo contains decreased expression levels of miR-24-2 as compared to N-exo) which improved after transfection with miR-24-2 miRs. N-exo treated HUVECs showed regular cobblestone morphology (as it is seen, N-exo contains increased levels of miR-24-2 as compared to R-exo) which deteriorated after transfection with miR-24-2 anti-miRs (Figure 11).

Figure 11. Effect of treatment of miR-24-2 specific- miRs and anti-miRs in exosomes treated HUVECs: Representative micrograph of HUVECs treated by (a) scrambled miR+PBS, (b) R-exo, (c) R-exo+miR-24-2-miRs, (d) N-exo and (e) N-exo +miR-24-2 anti-miRs (10X). Scale bar = 400 µm.

Further, HUVECs treated with N-exo transfected with miR-24-2 anti-miRs, showed significantly reduced expression levels of exosomal miR-24-2 ($P<0.01$) as compared to N-

exo treated-HUVECs; whereas the expression levels of SMA-α (mesenchymal marker) (P<0.01) and TGF-β
(P<0.001), significantly enhanced in miR-24-2 anti-miR transfected HUVECs treated with N-exo as compared to HUVECs treated with N-exo. The endothelial marker (VE-cadherin) (P<0.001) showed a similar trend in expression levels of miR-24-2. Nevertheless, HUVECs treated with R-exo transfected with miR-24-2 miRs, showed significantly increased expression levels of miR-24-2 (P<0.001)as compared to R-exo treated-HUVECs; whereas, the levels of TGF-β (P<0.05) and SMA-α (P<0.001) significantly decreased in HUVECs treated with R-exo transfected with miR-24-2 miRs as compared to HUVECs treated with R-exo. However, VE-cadherin levels significantly increased (P<0.001) in HUVECs treated with R-exo transfected with miR-24-2 miRs as compared to HUVECs treated with R-exo (Figure 12), thereby, indicating that EndoMT and elevation in the expression levels of TGF-β (indicative of fibrosis) takes place in exosomes treated-HUVECs.

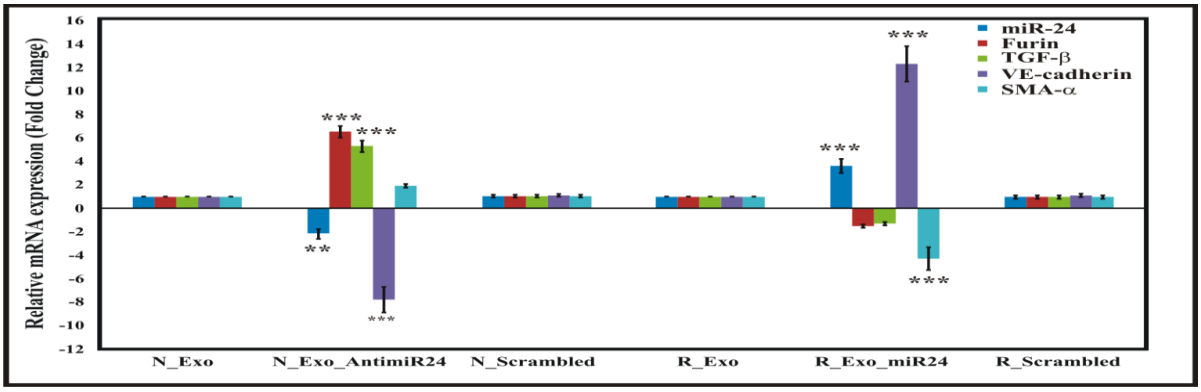


Figure 12. Gene expression levels after transfection with miR-24-2 specific-miRs and anti-miRs transfection in exosomes treated HUVECs: RT PCR of miR-24-2, its target genes (furin), downstream gene (TGF-β), and endothelial marker (VE-cadherin) and mesenchymal marker (SMA-α) in HUVECs treated with scrambled siRNA, R-exo, R-exo+ miR-24-2 miRs and N-exo and Nexo + miR-24-2 anti miRs, as compared to respective controls. All experiments were repeated three times. Each bar represents the mean ± S.D. Statistical analysis was carried out by oneway ANOVA. **P < 0.01, ***P < 0.001compared to control

Protein levels of SMA-α, TGF-β & VE-cadherin in exosomes treated HUVECs and mitral valve tissues of RHD patients

Further, the protein levels for TGF-β, SMA-α and VE-cadherin were checked in R-exo and N-exo treated HUVECs and mitral valve tissues of RHD patients. It was observed that the protein levels of TGF-β (P<0.001) and SMA-α (P<0.001) were significantly augmented whereas the protein levels of VE-cadherin were reduced in R-exo treated HUVECs (P<0.05) and mitral valves of RHD patients (P<0.05) as compared to their respective controls (Figure 13), thereby, suggesting that EndoMT occurs in ECs lining the mitral valves of the heart and consequently leads to mitral valve fibrosis during RHD.

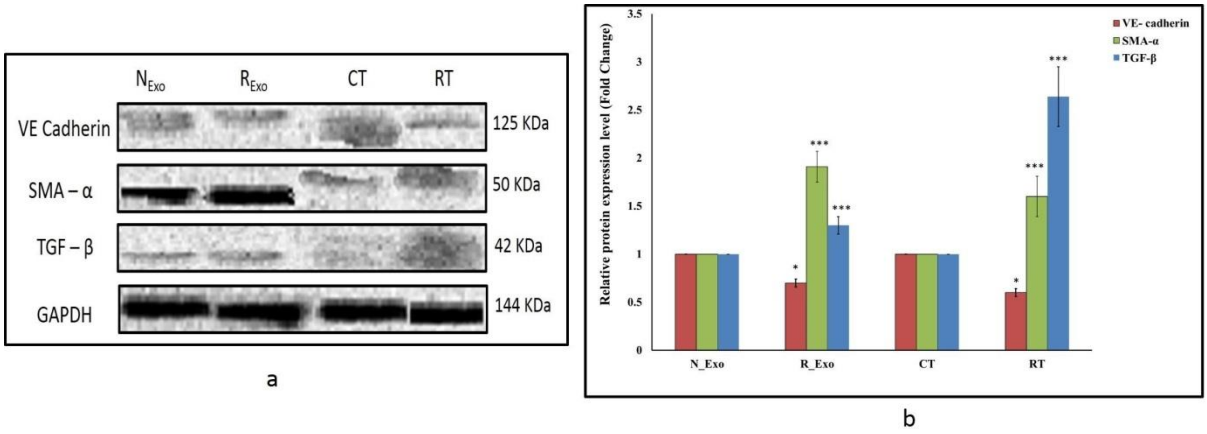


Figure 13. Protein levels of miR-24-2 downstream gene, TGF-β and endothelial (VE cadherin) and mesenchymal markers (Sma-α): Western blot analysis for VE-cadherin, SMA-α and TGF-β protein in R-exo and N-exo treated HUVECs and mitral valve tissues of RHD patients (RT) and controls(CT) (a) Representative western blot images (b) Relative levels of protein levels normalized to GAPDH. All experiments were repeated three times. Each bar represents the mean ± S.D. Statistical analysis was carried out by student's t-test. *P < 0.05, ***P < 0.001compared to control**.**

Haematoxylin-Eosin (H& E) and Mason trichrome (MT) staining of mitral valve tissues:

Haematoxylin-Eosin and mason trichrome staining of mitral valves tissues showed more hyalinization (indicative of fibrosis) of the mitral valve tissues obtained from RHD patients as compared to the control tissues (Figure 14), thereby, suggesting that mitral valves fibrosis occurs during RHD.

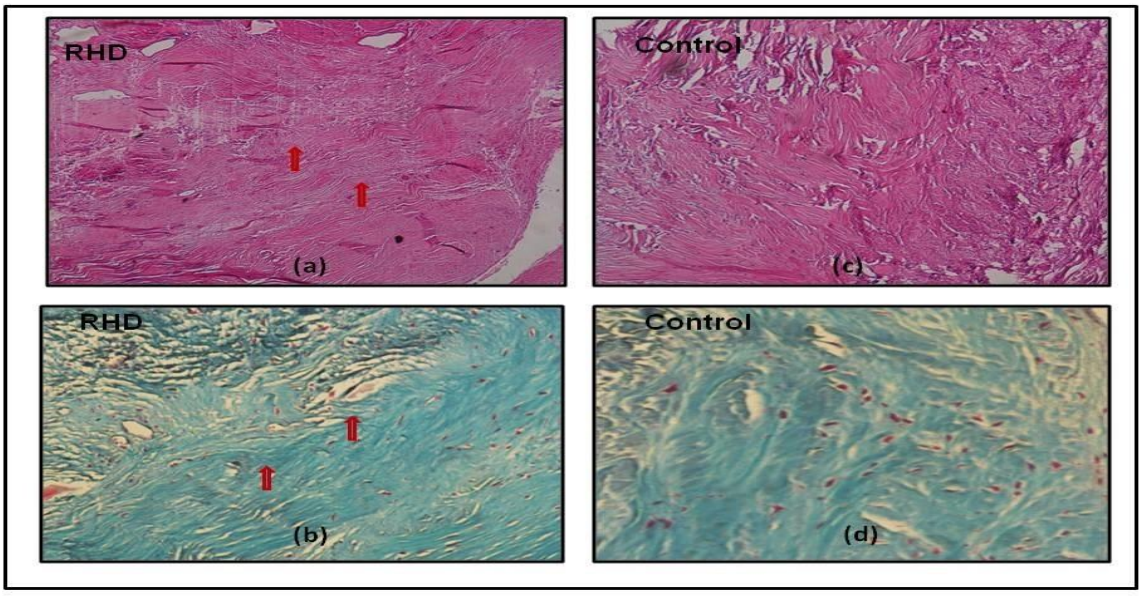


Figure 14: Haematoxylin-eosin and mason trichrome (HE and MT) staining of mitral valve tissues: Representative images of (a,c) HE and (b,d) MT stained mitral valve tissues from RHD patients as compared to controls, respectively showing hyalinization in the tissues

4. Discussion

Rheumatic heart disease is an autoimmune disease that leads to permanent mitral valves damage**.** The mitral valves are lined by endothelial cells and these endothelial cells undergo endothelial to mesenchymal transition. The mesenchymal cells are fibroblasts and during mechanical injury or stressed/diseased condition, the fibroblasts differentiate into myofibroblasts and produce transforming growth factor (TGF-β) and production of TGF-β, leads to tissue fibrosis during diseased conditions **[3]**. Recently, exosomes have been known to play a vital role in various cardiovascular diseases **[5].** In the present study, the role of exosomes has been unfurled in mitral valves fibrosis and dysfunction during RHD pathogenesis. HUVECs treated with RHD patient's serum exosomes (R-exo) and normal individuals' serum exosomes (N-exo), revealed that R-exo treated HUVECs undergo EndoMT where mesenchymal cells (fibroblasts) formed mostly express significantly increased expression levels of mesenchymal cell markers (SMA-α) as compared to endothelial cell markers (VE-cadherin). Interestingly, it was important to understand whether the miRNA content of exosomes, is responsible for the mitral valve dysfunction during RHD. Various studies have suggested the role of miR-24-2 during cardiac fibrosis **[12,13]** and exosomal miRNA sequencing analysis, also revealed miR-24-2 (down-regulated exosomal miR) to be the common miRNA in serum and PF and media of RHD *in-vitro* model. The endothelial cells line the mitral valves, which is the site where auto-antibodies binds during autoimmune response generated during RHD, therefore, HUVECs were isolated and stimulated with pooled RHD patients' serum to generate RHD *in-vitro* model which in-order to mimic the *in vivo*-RHD conditions in endothelial cells of the mitral valves of heart. So, the significant down-regulated expression of exosomal miR-24-2 has been found in exosomes of biological fluids (serum, PF)-*patient samples*, which can be correlated with RHD *in-vitro* model conditions, as the exosomes in media of RHD *in-vitro* model also showed the significantly decreased expression of exosomal miR-24-2 as compared to HUVECs without stimulation. The expression of exosomal miR-24-2 was even found to be significantly reduced in R-exo-treated HUVECs and mitral valves tissues of RHD patients.

Mitral valve fibrosis is the outcome of RHD, therefore the study was mainly focused on the evaluation of gene expression responsible for causing mitral valves fibrosis. Therefore, miR-24-2 target gene, furin (TGF-β activator) and downstream TGF-β gene expression levels (downstream pathological mediator of cardiac fibrosis) were estimated in the mitral valve tissues, where it was observed that the expression levels of furin and TGF-β were significantly elevated. Finally, functional inhibition of miR-24-2 was performed in exosomes-treated HUVECs, which showed significantly augmented expression levels of SMA-α (mesenchymal cell marker) and TGF-β (fibrosis marker) and declined levels of VE cadherin in R-exo treated HUVECs as compared to N-exo treated HUVECs, thereby, confirming that EndoMT takes place and enhances the levels of TGF-β (indicative of fibrosis) in exosomes treated HUVECs. Further, protein levels of TGF-β, SMA-α and VE cadherin were checked in the mitral valve tissues of RHD patients, which showed altered protein levels of these markers, that suggested the role of exosomal miR-24-2 in EndoMT in HUVECs which can be correlated with EndoMT in endothelial cells lining the mitral valves of the heart, eventually leading to mitral valves fibrosis and dysfunction during RHD.

To sum up, the exosomes in the biological fluids (serum and PF), are taken up by mitral valve endothelial cells during RHD. The exosomes in biological fluids of RHD patients contain very low levels of miR-24-2. Hence, reduced levels of exosomal miR-24-2 are taken up by the recipient cells (endothelial cells of the mitral valves), which leads to endothelial to mesenchymal cell transition in the valves. Myo-fibroblasts (mesenchymal cells) formed, elevates the levels of downstream TGF-β. Furthermore, declined exosomal miR-24-2 levels in mitral valve tissues of RHD patients, leads to increase in expression levels of its target gene, furin, which in turn, activates its downstream gene, TGF-β, expression levels, consequently, leading to mitral valves fibrosis and dysfunction (stenosis and regurgitation) during RHD.

5. Conclusion

In conclusion, in the present study, the role of exosomal miR-24-2 in causing mitral valves fibrosis and dysfunction during RHD pathogenesis, has been demonstrated, hence, declined expression levels of exosomal miR-24-2 is indicative of predicting severity during RHD and can open doors to the future research to better understand the disease.

Availability of Data and Material: Yes

Author's Contribution: AC conceived the idea of the study. SS, designed the framework, performed experimental work and drafted the manuscript, HS, SC & UN provided the samples for the study and gave their critical comments. All authors approved the final version of the manuscript.

Code Availability: None

Funding: The funding was received by Indian Council of Medical Research (ICMR), Delhi, India.

Compliance with Ethical Standards: Yes

Conflicts of Interest/Competing Interests: None.

Ethics Approval: Approval for the study was granted by Institutional Ethics Committee, Postgraduate Institute of Medical Education and Research, Chandigarh, (INT/IEC/2017/1453; Date: 04.12.2017).

Consent to Participate: Informed consent was obtained from all individual participants included in the study.

Consent for Publication: Yes

Acknowledgements: This study was supported by grants from Indian Council of Medical Education (ICMR). We thank the professors from different departments for providing the samples and for editing the manuscript, and also the technical staff for their excellent technical assistance.

References:

[1] Liu M, Lu L, Sun R, Zheng Y, Zhang P. Rheumatic Heart Disease: Causes, Symptoms, and Treatments. Cell Biochem Biophys.. 2015; 72:861-63.

[2] Roberts S, Kosanke S, Dunn ST, Jankelow D, Duran CMG, Cunningham MW. Immune mechanisms in rheumatic carditis: Focus on valvular endothelium. J Infect Dis.2001; 183:507-11.

[3] Porter KE, Turner NA. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol Ther. 2009; 123:255-78.

[4] Carapetis JR, Beaton A, Cunningham MW, Guilherme L, Karthikeyan G, Mayosi BM, et al. Acute rheumatic fever and rheumatic heart disease. Nat Rev Dis Primers. 2016; 2:15084.

[5] Tian C.Gao L, Zimmerman MC, Zucker IH.. Myocardial infarction-induced microRNA-enriched exosomes contribute to cardiac Nrf2 dysregulation in chronic heart failure. Am. J. Physiol. Heart Circ. Physiol. 2018; 314: 928-39.

[6] Simons M, Raposo G. Exosomes Vesicular carriers for intercellular communication. Curr. Opin. Cell Biol.2009; 21: 575-81.

[7] Caradec J, Kharmate G, Hosseini-Beheshti E, Adomat H, Gleave M, Guns E. Reproducibility and efficiency of serum-derived exosome extraction methods. Clin. Biochem. 2014; 47:1286-92.

[8] Ha M, Kim VN. Regulation of microRNA biogenesis.. Nat Rev Mol Cell Biol.2014; 15:509-24.

[9] Broughton JP, Lovci MT, Huang JL, Yeo GW, Pasquinelli AE. Pairing beyond the Seed Supports MicroRNA Targeting Specificity. Mol Cell. 2016; 64:320-33.

[10] Vasudevan S. Posttranscriptional upregulation by microRNAs. Wiley Interdiscip Rev RNA.2012; 3:311-30.

[11] Makarova JA, Shkurnikov MU, Wicklein D, Lange T, Samatov TR, Turchinovich AA, et al. Intracellular and extracellular microRNA: an update on localization and biological role. Prog Histochem Cytochem. 2016; 51:33-49.

[12] Suzuki HI, Katsura A, Mihira H, Horie M, Saito A and Miyazono K.. Regulation of TGF-b-mediated endothelial-mesenchymal transition by microRNA-27.J. Biochem. 2017; 161(5):417–420

[13] Wang J, Huang W, Xu R, Nie Y, Cao X, Meng J et al. MicroRNA-24 regulates cardiac fibrosis after myocardial infarction. J Cell Mol Med.2012; 16:2150-60.

[14] Baudin B, Bruneel A, Bosselut N, Vaubourdolle M. A protocol for isolation and culture of human umbilical vein endothelial cells. Nat Protoc.2007; 2:481-85.

[15] Rastogi M, Sarkar S, Makol A, Sandip Singh R, Saikia UN, Banerjee D, et al. Anti-endothelial cell antibody rich sera from rheumatic heart disease patients induces proinflammatory phenotype and methylation alteration in endothelial cells. Genes Dis.2018; 5:275-289.

[16] Qu Q, Pang Y, Zhang C, Liu L, Bi Y. Exosomes derived from human umbilical cord mesenchymal stem cells inhibit vein graft intimal hyperplasia and accelerate reendothelialization by enhancing endothelial function. Stem Cell Res Ther.2010;11:133.

[17] Wong N, Wang X.miRDB: an online resource for microRNA target prediction and functional annotations. Nucleic Acids Res.2015;43:D146-52.

[18] Chen Z, Lu S, Xu M, Liu P, Ren R, Ma W. Role of miR-24, Furin, and Transforming Growth Factor-β1 Signal Pathway in Fibrosis after Cardiac Infarction. Med Sci Monit. .2017;23:65-70.

[19] Lu Q, Sun Y, Duan Y, Li B, Xia J, Yu S *et al*. Comprehensive microRNA profiling reveals potential augmentation of the IL1 pathway in rheumatic heart valve disease. BMC Cardiovascular Disorders;2018. 18:53.