RESEARCH PAPER

MiR-24-2 in exosomes elevate TGF-β levels and mediate mitral valve dysfunction during rheumatic heart disease pathogenesis

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Abstract

Background & Objective: Rheumatic heart disease (RHD) is an autoimmune sequel of rheumatic fever (RF) that leads to permanent damage to the mitral valves. Endothelial cells (ECs) that line the mitral valves, take up exosomes from the biological fluids, which change the cellular characteristics of ECs, resulting into mitral valves fibrosis. Herein, an attempt has been made to evaluate the role of exosomal miR-24-2 in mitral valve damage during RHD.

Methods: The present study was divided into: *in- vitro* set-up and *patient samples* analysis where, the exosomes were isolated *from patient samples* (serum and pericardial fluid) of RHD patients and simultaneously from the *media* extracted from RHD patients' serum stimulated HUVECs (RHD- *in-vitro* model). Further, HUVECs were treated with exosomes from RHD patients' serum (R-exo) and normal individuals' serum (N-exo) in-order to evaluate biological changes in the cells. Next, miRNA profiling and RT-PCR was performed, to check de-regulated miRNAs and its target gene.

Results: As findings, significantly declined levels of miR-24-2 was observed, with increased furin (target gene) and TGF-β levels in the mitral valves of RHD patients. Finally, to check the cause and effect, functional inhibition of exosomal miR-24-2 was done in exosomes-treated HUVECs, where significantly decreased gene and protein expression levels of endothelial marker (VE cadherin), and simultaneously, increased levels of mesenchymal (SMA-α) and fibrosis marker (TGF-β) were observed in R-exo-treated HUVECs as well as mitral valves of RHD patients.

Conclusion & interpretation: These findings suggest that decreased exosomal miR-24-2 levels lead to enhanced TGF-β production, which is responsible for mitral valves dysfunction during RHD.

Keywords: RHD Exosomes miRNA Fibrosis EndoMT

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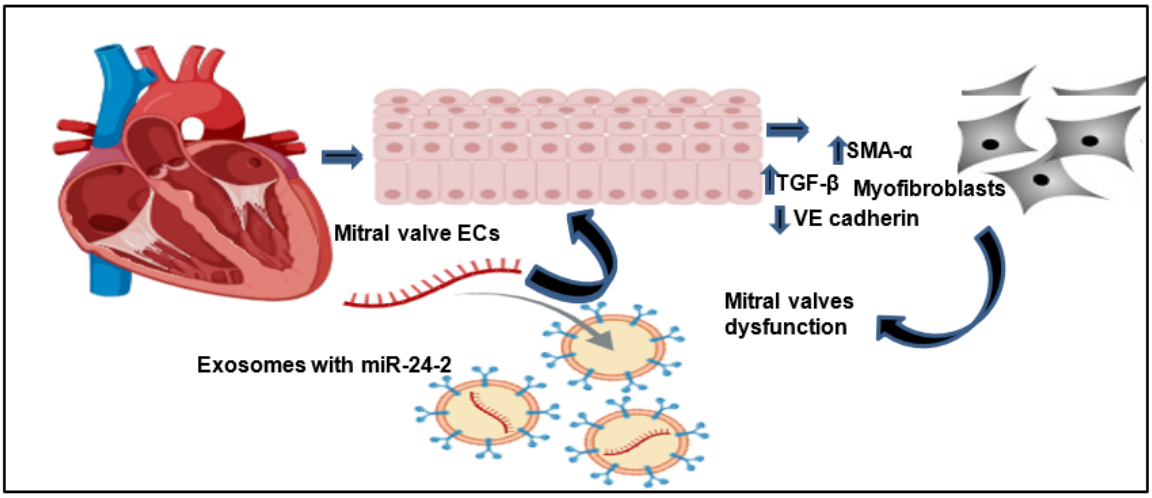
Graphical abstract



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. A single severe episode of rheumatic fever or recurrent events of rheumatic fever (RF) results in permanent mitral valves damage **[1].** It occurs mostly in children and may lead to lifelong disability or death**.** According to WHO report, 2025, 55 million people worldwide suffer from RHD, which results in about 360,000 fatalities annually. Molecular mimicry theory (MMT) is the mechanism, which is responsible for RHD pathogenesis. According to this theory, there exists a structural similarity between Group A streptococcus proteins and cardiac proteins, hence, autoantibodies produced against GAS, cross-react with cardiac proteins and attacks particularly, the mitral valve endothelium, that leads to increased expression of cell adhesion molecules like vascular cell adhesion molecule-1 (vCAM-1) and intercellular cell adhesion molecule (iCAM). The immune cellular infiltration takes place in the mitral valves, leading to inflammation, which results in initial scarring in the mitral valve tissues, and eventually progresses into RHD **[2].** Fibroblasts (mesenchymal origin), which differentiate into myo-fibroblasts, are responsible for tissue fibrosis. Also, diseased conditions lead to the release of cytokines by fibroblasts, such as TGF-β, which enhances fibrosis during this any pathological conditions.**[3].** Mitral valve fibrosis is the hallmark of RHD. **[4].** It is therefore important to uncover the causative factors which lead to mitral valves fibrosis, in-order to better understand the disease.

Nowadays, research has been focused on exosomes, which play an important role in various physiological and pathological cardiovascular processes **[5].** Exosomes, the nano-vesicles, (30-150 nm in size) are released by different cell types **[6]**, and are found in various body fluids such as serum, plasma, breast milk, saliva, amniotic fluid, urine, and also in cell culture media **[7].** Exosomes are found to be abundant in bio-molecules, such as RNAs, microRNAs, DNAs, lipids, and proteins. In addition, by transfer of these bioactive cargos to recipient cells (target cells), these vesicles act as cell-to-cell communicators. Thus, exosomal miRNAs have proven to play a crucial role in promoting or preventing various diseases.

MicroRNAs are small non-coding RNAs that 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 transcription **[11].**

RHD pathogenesis is still unclear. Various studies have indicated the emerging role of miR-24-2 is in EndoMT and fibrosis during cardiac diseases **[12,13].** However, the role of exosomal miRNA-24-2 in RHD pathogenesis, has not been documented yet. Hence, in the present study, the role of exosomal miRNA-24-2 in mitral valve damage has been investigated to better understand the obscure pathogenesis of this rare disease.

Materials and methods

Collection and processing of samples

The present study was ethically approved by Institutional Ethics Committee (INT/IEC/2017/1457). 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 (MVR) surgery 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 (n=42) were obtained from healthy individuals, and mitral valve tissues (n=20) were collected from cadavers with no pathological, morphological heart disease or any inflammatory disease. Post-mortem interval (PMI) was minimized and standardised tissue handling was done to avoid degradation of biomolecules, as standardized in our laboratory. Furthermore, cadaver donar's demographic details were matched with patients' demographic details. 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 (100–150 mg) were collected in phosphate buffer saline (PBS) and brought to the laboratory on 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 the samples were stored at -80°C until further use. After removal of debris from PF, the samples were centrifuged at 4000 g for 15 min and were stored at -80°C until further use.

Isolation, culture and characterization of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord **[14].** Briefly, the cord was brought to the laboratory in 1X PBS containing penicillin (1million units/50 ml) at 4°C. The cannula was inserted into the vein and washed with PBS once. Collagenase (0.2%,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 a Neubauer chamber (Marinfeld, Germany) and thereafter, cells were seeded on a 96-well plate. HUVECs were grown on 96-well plates till 50–60% confluency. Fixing of cells was carried out with 4% paraformaldehyde in PBS at RT, followed by two washes with PBS and blocking with 5% Bovine serum albumin (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.

Development of RHD *in-vitro* model (Stimulation of HUVECs with RHD patients' pooled serum)

Cells were grown to 70–80% confluency, and RHD patients' pooled serum was added to the cells in a 12-well plate to a final dilution of 1:5, 1:50, and 1:100 for 6 h and 12 h in a 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) associated with RHD pathogenesis was carried out to confirm the development of RHD *in-vitro* model **[15].** For the collection of media, HUVECs were cultured at 90–100 %, density in medium with 10% exosome-depleted FBS (Gibco, USA) for 72 h. The medium was centrifuged for 10 min at 2000 × g, then for 30 min at 10,000 × 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 exosome precipitation reagent at a ratio of 4:1 followed by overnight incubation at 4˚C. Further, centrifugation was carried out at 500 × g for 5 min for serum and 10,000 × g for 30 min for PF and media. Next, exosome pellets were resuspended in PBS (GE Healthcare Life Sciences, USA). The exosome pellets were used at once or stored at -80˚C till further use.

Transmission Electron Microscopy (TEM)

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

Zeta sizing

Zeta sizing (Malvern Panalytical, UK) was outsourced from NIPER, Mohali, India. By using the technique of Dynamic Light Scattering (DLS), zeta sizer was used to measure the particle size, diameter, and percentage intensity of exosomes, according to the operating instructions. The sample was prepared by mixing exosomes in PBS at 1:400 dilution.

Western blot

Western blot was performed for the proteins isolated from HUVECs and exosomes. HUVECs and exosomes were lysed in RIPA buffer (ice cold). The protein concentration was measured by the bicinchoninic acid (BCA) method. The protein samples of 50 µg were loaded on SDS-PAGE and the protein bands from the gel were transferred (80V, 2.5 h, 4ºC) to Polyvinylidene fluoride membrane (PVDF) (GE Health care, USA) in the transfer buffer (25 mM Tris/192 mM Glycine (pH 8.0)/20% methanol) using mini-tank transfer unit (Bio-Rad mini Transblot® Cell, Bio-Rad, USA). The blocking of membrane was done with 5% skimmed milk in Tris-buffered saline-0.1% Tween 20 (TBST overnight at 4ºC), after transfer of protein bands to PVDF membrane. The membrane was washed three times, 5 min each, with TBST to remove unbound blocking reagent. Primary antibody was added against CD- 63, (Biovision, USA, 1:100), TGF-β (Biovision, USA, 1:200), SMA-α (Biovision, USA, 1:200), VE cadherin (Biovision, USA, 1:100), and β-actin (Biovision, USA, 1:200) and incubated at 37ºC with shaking. The 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,

the washing steps were repeated. The membrane was exposed for one min or longer for visualization and the images were acquired in Protein Simple FlourChem M (Bio-Techne, USA). Densitometric quantification of the bands was done using Image J.

Exosome uptake in HUVECs

In order to confirm exosome uptake by HUVECs, an exosome uptake experiment was performed by labeling exosomes with PKH-67 dye (Sigma, USA), according to manufacturer's instructions. The dye labels the lipophilic membrane of the exosomes. Briefly, PKH-67 dye (2 µl) was diluted in 100 µl diluent C and incubated at 37°C for 15 min. Exosomes (10 µl) were 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% exosome-depleted FBS was added, and the mixture was filtered with Amicon filters 10kDa (Merck, USA). Labelled exosomes were collected and added to HUVECs, and the cells were kept at 37°C for 24 h in 5% CO_2 incubator. HUVECs were viewed under a 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 h, to evaluate the role of exosomes in HUVECs, after determination of exosome 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 ($2 \times 10_4$) 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 was added to each well. Cells were incubated for 2 h 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, by centrifugation, 5×10^5 cells were collected and dissolved in 1X Binding buffer (500 µm µl), and Annexin V-FITC (5 µl) and propidium iodide (5 µl) were added. Cells were incubated for 5 min in the dark at RT. Finally, analysis of Annexin V-FITC binding was performed 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 exosome-treated HUVECs. To conduct matrigel assay, HUVECs were grown up to 90% confluency at 37°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°C to allow the solution to form the gel. Approximately 1×10^4 endothelial cells (ECs) were seeded using 100 µl culture medium. For inhibitor control (suramin) wells, same number of cells were kept in an 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 h at 37°C in a CO_2 incubator, and the images were captured under an 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 (Qiagen, 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, using the following primers, for further use **(Supplementary: Table 1).**

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 ($Cq \geq 2$) and the miRNA with the role in cardiac fibrosis and EndoMT. MiRNA-24-2 specific qRT-PCR was performed for exosomal RNA isolated from biological samples (serum and pericardial fluid), N-Exo and R-Exo treated HUVECs, and mitral valve tissues. RNA was reverse-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 was used to calculate fold change (Roche, Germany). The PCR conditions were as follows: holding stage: 95˚C for 180 s; cycling stage: 95˚C for 15 s and 60˚C for 30 s, 40 cycles

Target gene prediction

Furin was the predicted target gene of miR-24-2, which was analysed by using miRDB software. Nevertheless, an extensive literature search was also performed to find out the target gene for miR-24-2 **[17].** HUVECs were transfected with miRs and anti-miRs specific for the selected miRNA- 24-2. Next, miRNA and target gene (furin) expression were evaluated. Furin enhances the levels of downstream TGF-β during cardiac fibrosis via the furin-TGF-β pathway **[18].** Furin (latent activator of TGF- β) 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 exosome-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 the same media, without antibiotics. Lipofectamine RNAiMAX reagent and siRNA were mixed in 1:1 ratio and incubated for 5 min at RT. For forward and reverse transfection, 2×10^5 cells were seeded in a 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 was observed, followed by trypsinization and RNA isolation. The gene expression analysis for selected miRNA-24-2 and its target gene, furin, was also carried out. Further, EndoMT markers (VE cadherin and SMA-α) and fibrotic genes (TGF-β) gene expression levels were also evaluated.

Haematoxylin-Eosin staining of mitral valve tissues

To check for mitral valve fibrosis, haematoxylin-eosin staining was performed using standardized protocol. The slides with sections were placed in a metal staining rack. The sections were immersed in the filtered Harris Hematoxylin for 10 s. The tap water was exchanged until the water was clear. The sections were immersed in eosin stain for 30 s. 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 valve tissues

To confirm, mitral valve fibrosis, fibrosis-specific staining procedure was performed using mason trichrome staining method by using standardized protocol. Weigert's iron hematoxylin stains the nuclei in black, Biebrich scarlet-acid fuchsin stains cytoplasm and 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 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, Bonferroni and Dunnett post-hoc test) was used to analyse data from more than two groups. Statistical software SPSS was used for assessing data. A p-value of 0.05 was considered statistically significant.

Results

Human umbilical cord vein endothelial cells culture: HUVECs were cultured in endothelial cell growth medium (Sigma Aldrich, USA) and maintained up to 4 days. The cultured HUVECs showed cobblestone

morphology (Fig. 1A) with oval nuclei and irregular cell shape throughout the experiment. The isolated cells were HUVECs, identified by indirect immunofluorescence, using an antibody against endothelial cell surface marker CD-31. The overlapped images of DAPI and PE labelled CD-31, suggested that the isolated cells were HUVECs (Fig. 1B), and these cells were taken for further analysis.

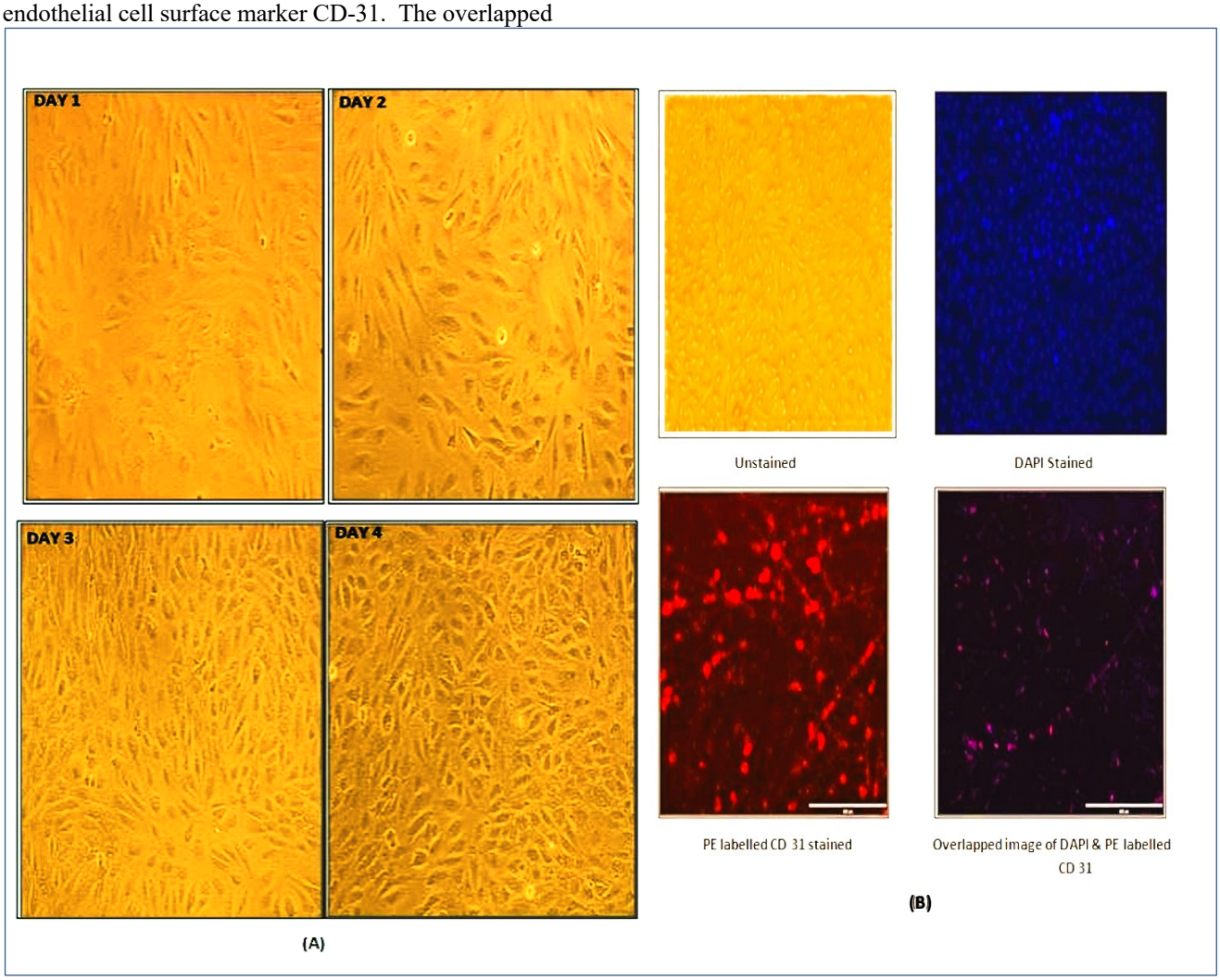


Fig 1 (A) Human umbilical cord vein endothelial cells (HUVECs) culture and maintenance: The isolated HUVECs were cultured and maintained in endothelial cell growth medium up to 4 days. **(B) Confirmation of isolated cells as HUVECs by indirect immuno-fluorescence:** Fluorescent immunostaining for endothelial cell surface marker CD-31, expressed by HUVECs, (10X). Scale bar = 400 µm.

Development of RHD *in-vitro* model

In order to develop RHD *in- vitro* model, HUVECs (primary cells) were stimulated by pooled RHD serum at three different dilutions, 1:5, 1:50, and 1:100, at different time intervals of 6 h and 12 h. HUVECs showed a rounded appearance at 1:50 and 1:100 dilutions at 6 and 12 h, more as compared than cells stimulated with pooled RHD patients' serum at 1:5 dilution for 6 and 12 h (Fig. 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 level of several genes: TNF-α ($p < 0.01$) INF-γ ($p < 0.01$), vCAM ($p < 0.001$), IL-1β ($p < 0.001$) was estimated which are known to be associated with RHD pathogenesis **[14]**.The expression levels of all the genes showed significant augmentation, that correlated with the development of RHD *in-vitro* model (Fig. 2B).

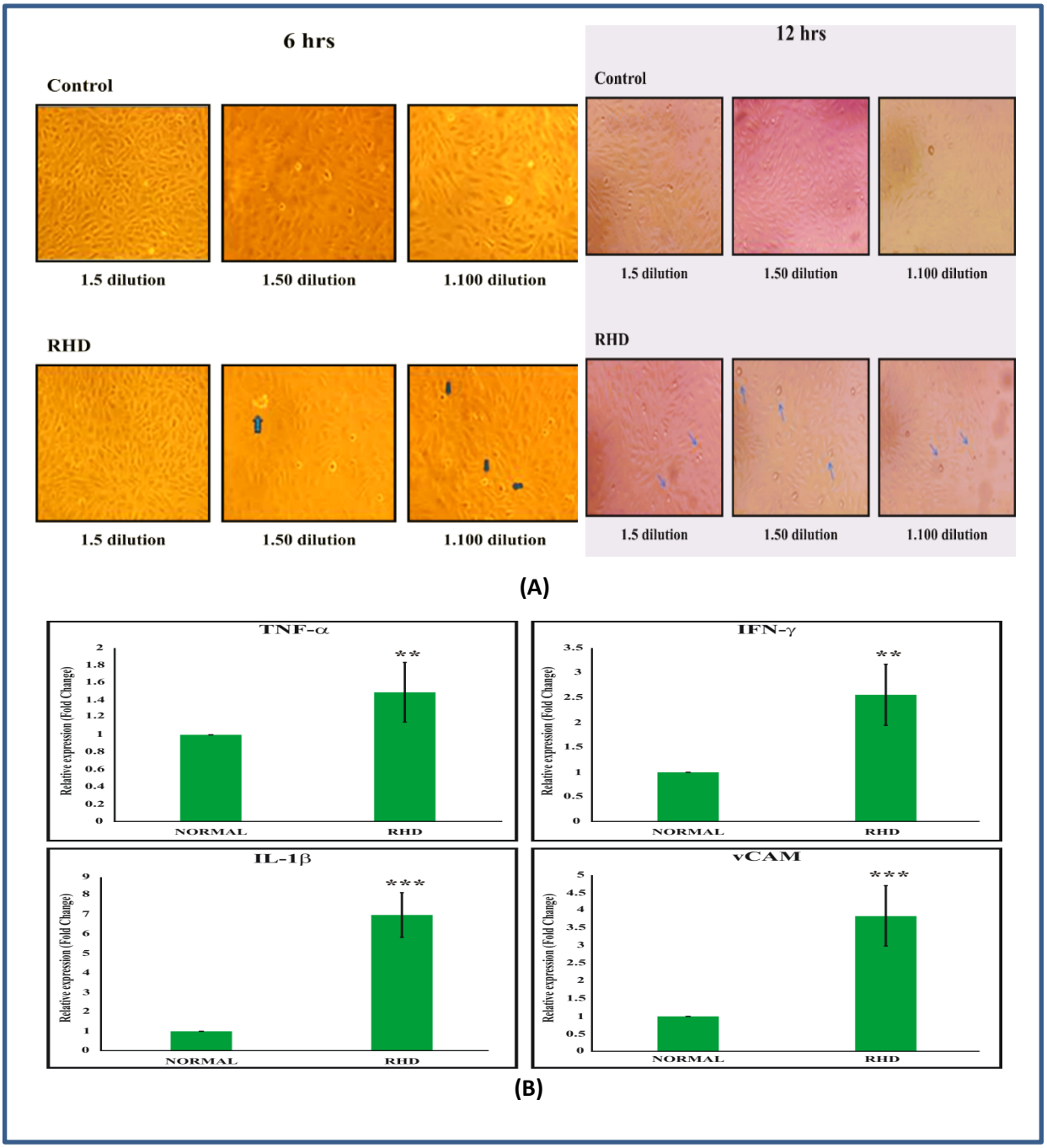


Fig 2 (A) Stimulation of HUVECs with pooled RHD patients' serum: Representative micrograph of HUVECs showing rounded bodies (arrow) upon stimulation with RHD patients' pooled serum at three different dilutions for 6 and 12 h. **(B) Relative gene expression of various pro-inflammatory genes and cell adhesion gene, 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). (10X). Scale bar = 400 µm. All experiments were repeated three times. Each bar represents the mean ± SD. Statistical analysis was carried out by Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control.

Isolation of exosomes

Exosomes were isolated from RHD patients' serum and PF samples. Simultaneously, exosomes were also isolated from stimulated HUVECs media (RHD *in-vitro* model) and their respective controls. The isolated exosomes were characterised using transmission electron microscopy (TEM), Zeta sizer, and western blot. In a study, by Sharma et al.2023, the exosomes were isolated and characterized to evaluate the role of de-regulated exosomal protein (alpha-1-antitrypsin) in RHD **[19].** In the present study, the exosomes were isolated and characterised to check the role of de-regulated exosomal

miRNA (miR-24-2) in RHD. TEM images confirmed a typical cup-shaped morphology, with the size range of 10–150 nm. The vesicles were further characterized by using zeta sizer to evaluate the percentage intensity of vesicles in different size ranges. Zeta sizing of isolated exosomes showed that 90% of vesicles were 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 (RHD *in-vitro* model and HUVECs without stimulation), respectively. Further, western blot analysis of isolated exosomes using exosomal surface marker CD-63 was performed, and a 65 KDa protein band specific to exosomal surface marker CD-63 was identified, which finally, confirmed that the isolated vesicles were exosomes (Fig. 3): published data.

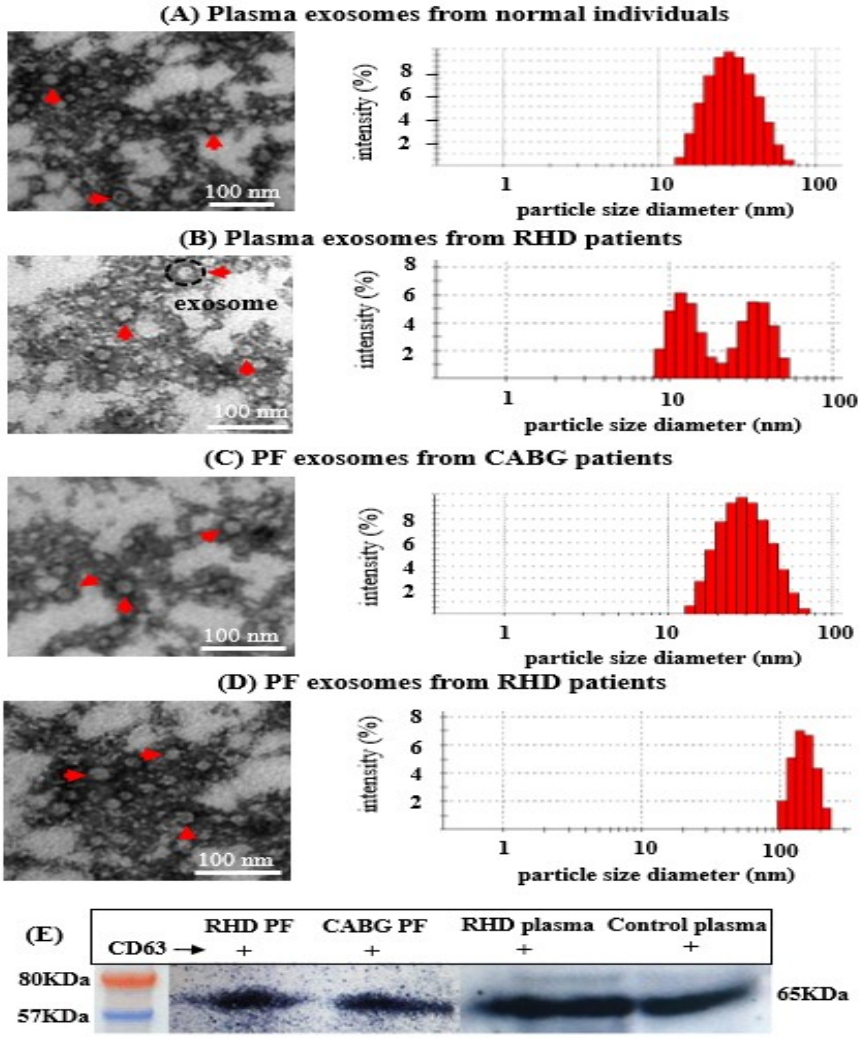


Fig 3 Characterization of exosomes from biological fluids from RHD patients and controls by TEM, Zeta Sizer (A-D), and Western Blot (E): published data ***"Reproduced with permission from Sharma et al. 2023 [19]. Copyright © 2022, The Author(s), under exclusive licence to Springer Science Business Media, LLC, part of Springer Nature."***

Effect of exosome treatment *in-vitro*

PKH-67 labels lipophilic membrane of the exosomes. Initially, PKH-67 labelled exosomes were collected and added to HUVECs, and the cells were kept at 37°C for 24 h 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 merged images, revealed the internalisation of the exosomes in HUVECs (Fig. 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 (component of serum) bring about any morphological changes at 1:10 dilution for 48 h, and it was observed that there was a distinct regular cup-shaped morphology seen in N-Exo-treated HUVECs, at 1:10 dilution for 48 h. However, rounded bodies could be seen in R-Exo-treated HUVECs, indicative of morphological changes associated with treatment of HUVECs with exosomes (Fig. 4B).

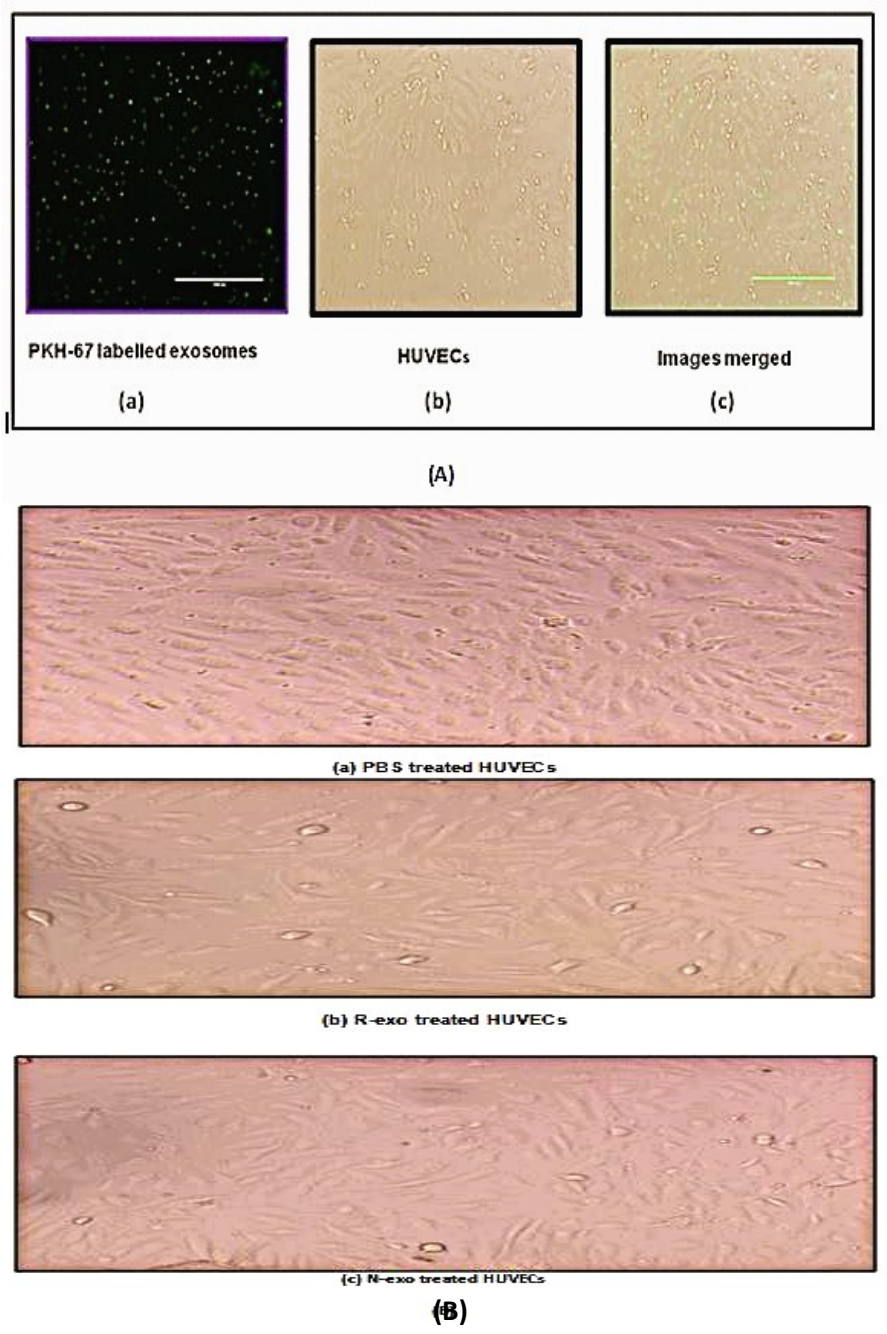


Fig 4 (A)Uptake of PKH-67-labeled exosomes by HUVECs: Representative images of (a) P

Figure 4 (A)PKH-67 labelled exosomes uptake by HUVECs: Representative images of (

Figure 4(A) PKH-67 labelled exosomes uptake by HUVECs: Representative images of a) PKH-67-labelled exosomes, (b) HUVECs, and (c) overlapped images showing exosome uptake by HUVECs.**(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 µ.

Next, changes in biological properties such as cell proliferation, apoptosis, angiogenesis, and expression levels of EndoMT markers were evaluated in exosome-treated HUVECs. The cell proliferation assay was conducted using a cell proliferation assay kit (Biovision, USA), and it was observed that HUVECs treated with R-Exos, significantly decreased ($p < 0.01$) in cell number as compared to HUVECs treated with N-Exos (Fig. 5A). Interestingly, 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 cells in R-Exo-treated HUVECs ($p < 0.01$) as compared to N-Exo-treated HUVECs (Fig. 5A). Next, the Matrigel angiogenesis assay was performed in N-

Exo- and R-Exo-treated HUVECs, using a tube formation assay kit specific for angiogenesis (Biovision, USA), where the beginning of angiogenesis was observed in N-Exo-treated HUVECs, whereas no angiogenesis was observed in R-Exo-treated HUVECs (Fig. 5B). Next, to confirm, these observations that angiogenesis occurs in N-Exo-treated HUVECs and apoptosis occurs in R-Exo-treated HUVECs, the gene expression of VEGF (angiogenic gene) and caspase-3 (apoptotic gene) in exosome-treated HUVECs was evaluated. 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, showing apoptosis takes place in R-exo treated HUVECs and angiogenesis takes place in N-exo treated HUVECs (Fig. 5C). These observations, thereby, indicates that the changes in morphology and biological properties in exosome-treated HUVECs. Further, the expression levels of mesenchymal markers (SMA-α) and endothelial cell markers (VE-cadherin) in N-Exo-treated HUVECs and R-Exo-treated HUVECs were checked. 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, thereby, suggesting that EndoMT takes place in R-exo treated HUVECs as compared to N-exo treated HUVECs. (Fig. 5C).

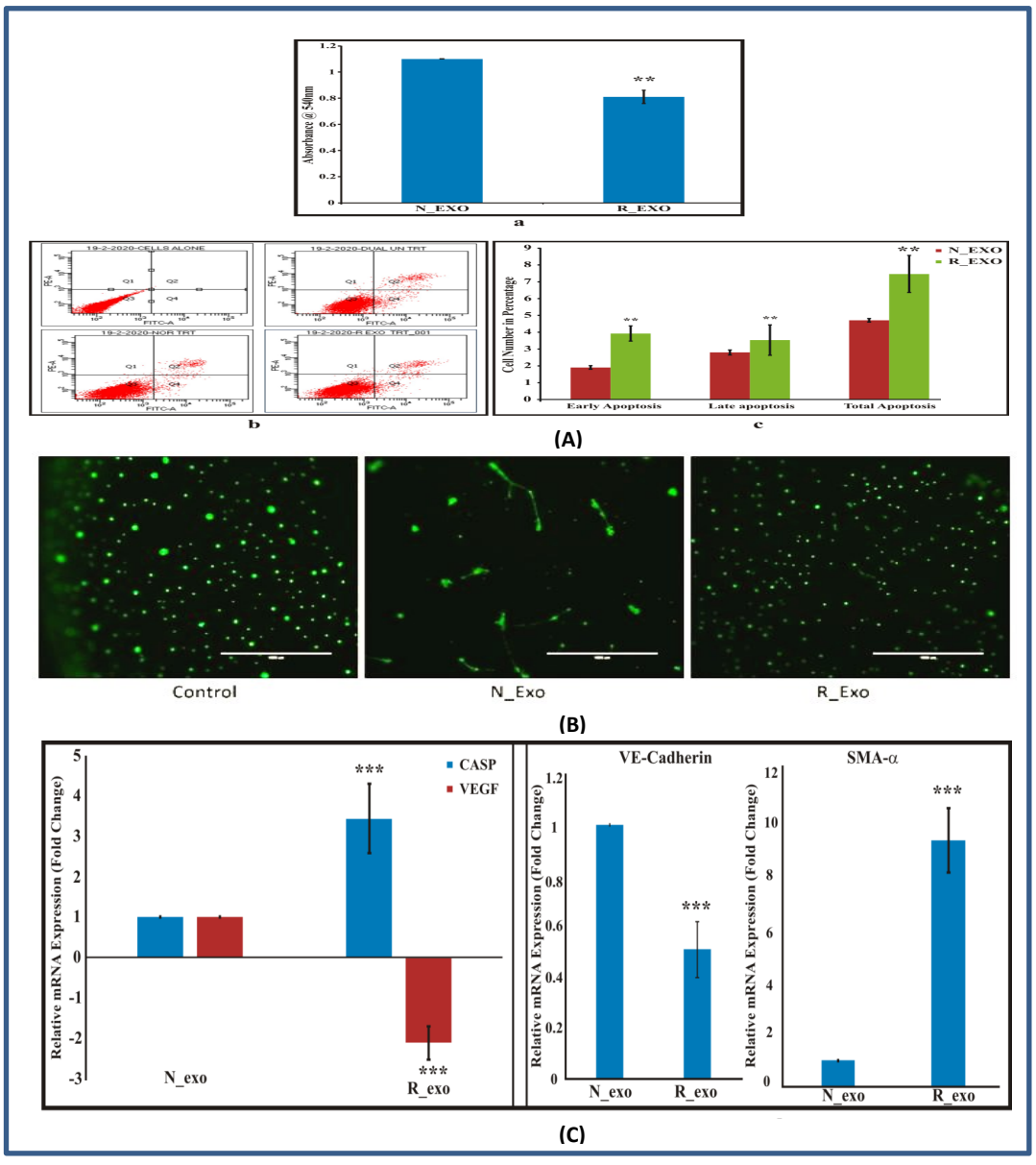


Fig 5 (A) Proliferative and apoptotic effect of treatment of exosomes in HUVECs: a) Cell proliferation assay showing relative cell number. **b)** The percentage of apoptotic cells was measured using the annexin V-PI assay by flow cytometry (Nor Trt = N-Exo treated, R Trt = R-Exo treated, DU Trt = dual untreated). **c)** Bar diagram representing the percentage of HUVECs undergoing apoptosis. **B)** The effect of angiogenesis after treatment of exosomes 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), **C)** Gene expression analysis of angiogenic, apoptotic, and EndoMT genes in exosome-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 ± SD. Statistical analysis was carried out by Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control.

Selection of exosomal miR-24-2

Initially, exosomes were isolated from serum and PF of RHD patients and simultaneously, with the media of stimulated HUVECs (RHD *in- vitro* model), including their respective controls. RNA was isolated from these exosomes and subjected to miRNA expression profiling, 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) was used to discover, identification, and expression of miRNAs based on latest miRBase release (v22). Differential expression analysis was done using edgeR (3.28.1). All heatmaps were generated from log (row count data) by using Complex Heatmap (version: 2.2.0). The gene chips represent different fold changes from many up-regulated miRNAs and down-regulated miRNAs by heat map of microRNA expression data (Supplementary Figs 1–3). A total of 2653 miRNAs were detected. Next, a common miRNA in all the heatmaps with ΔΔ Cq value ≥ 2, that revealed any association with EndoMT and cardiac fibrosis from previous studies, was selected as a criterion for selection of deregulated miRNA **[20],** Interestingly, exosomal miR-24-2 was screened as a common miRNA with down-regulated expression (ΔΔ Cq value ≥ 2) in serum, PF samples (in- vivo: patient-samples) and media (in-vitro RHD model). Next, extensive literature search was done, that showed the role of miR-24-2 in cardiac EndoMT and fibrosis **[12,13].** Exosomal miR-24-2 was screened as a selective miRNA matching the selection criteria, for further analysis. Next, target gene for miR-24-2 was evaluated using the target gene prediction miRDB software. Literature search and miRDB software analysis was performed where, furin was predicted as a target gene for miR-24-2. (supplementary Fig. 4).

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) and was known to be involved in EndoMT and cardiac fibrosis via literature search. Therefore, the expression levels of miR-24-2 were checked in serum exosomes, PF exosomes, R-Exo and N-Exo-treated HUVECs and mitral valve tissues of RHD patients. A total of 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 were 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 (Fig. 6A). 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 (Fig. 6B). MiR-24-2 influences the change in the levels of downstream gene TGF-β (fibrosis causing gene), via miR-24-2-furin-TGF-β pathway **[18].** Furin is a protease that controls the latent TGF-β activation process. The expression levels of furin and TGF-β (downstream gene, a pathological mediator that causes cardiac fibrosis) were 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 were 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 the expression levels of miR-24-2 were significantly reduced ($p < 0.001$) in the mitral valves of RHD tissues as compared to control tissues, indicating decreased levels of exosomal miR-24-2 uptake takes place, by the endothelial cells lining the mitral valves of RHD patients, which enhances the levels of furin and TGF-β in the tissues, leading to fibrosis. (Fig. 6C).

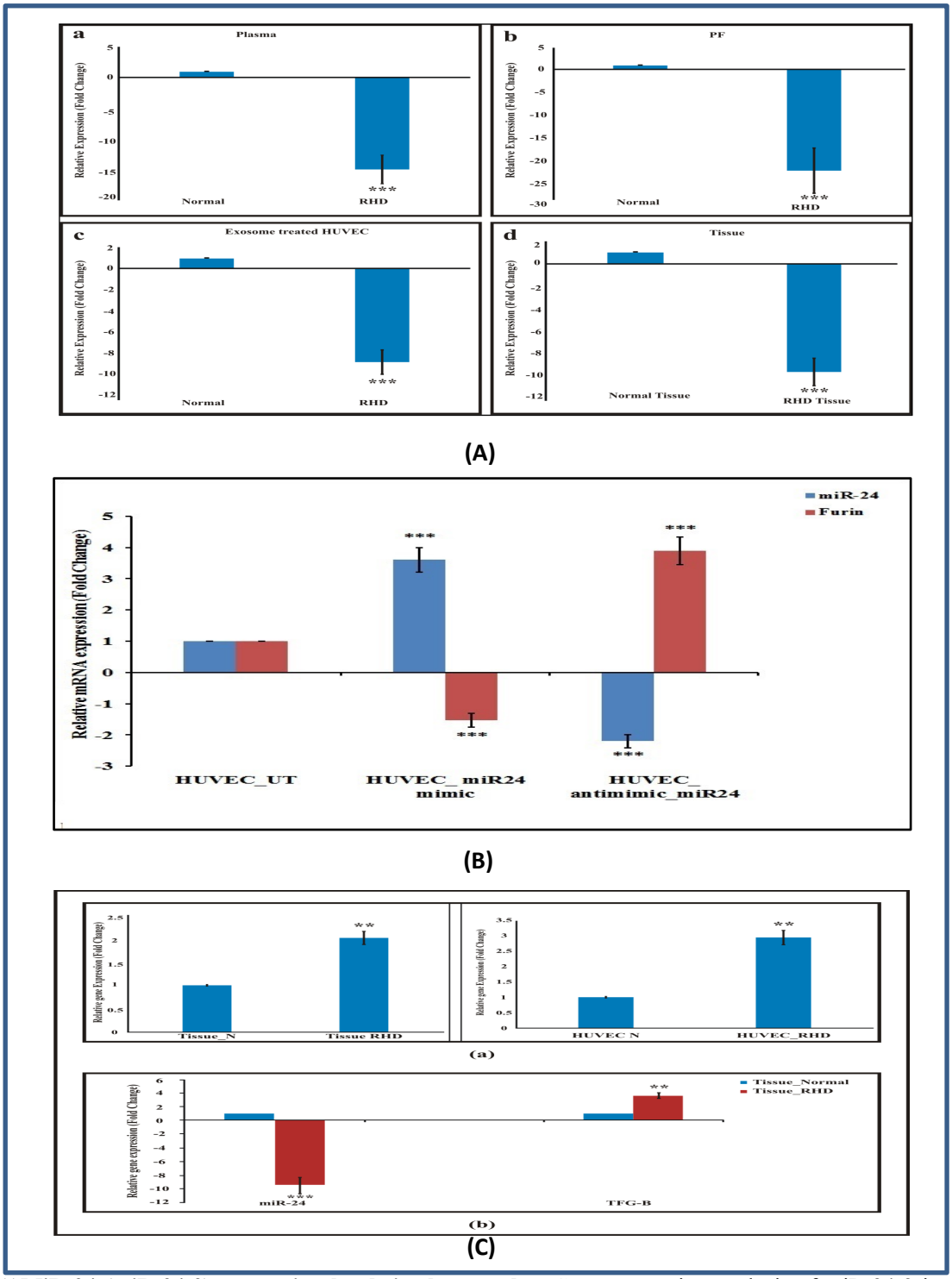


Fig 6 (A)MiR-24 (miR-24-2) expression levels in the samples: Gene expression analysis of miR-24-2 in (a) RHD patients' serum samples, (b) RHD patients' PF samples, (c) R-Exo-treated HUVECs, and (d) RHD patients' mitral valve tissues as compared to respective controls. **(B) MiR-24-2 target gene prediction by gene expression analysis:** Gene expression analysis of miR-24-2 and furin, after transfection of HUVECs with miR-24-2 miRs and anti-miRs. **(C) (a) Gene expression levels of exosomal miRNA-24-2 target gene (furin):** Gene expression analysis 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 miR-24-2 and downstream TGF-β: Gene expression analysis of miR-24-2 and TGF-β in mitral valve tissues from RHD patients 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 were analysed in five settings in HUVECs: R-Exo treated-HUVECs , R-Exo-treated-HUVECs transfected with miR-24-2 specific miRs, N-Exo treated-HUVECs, N-Exo-treated-HUVECs transfected with miR-24-2 specific 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 was confirmed in the previous observations, that R-Exo contains decreased expression levels of miR-24-2 as compared to N-Exo, which improved the morphology of HUVECs 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 (Fig. 7A). Further, cells were trypsinised and RNA was isolated from these cells. Interestingly, N-Exo- treated-HUVECs 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 N-exo- treated-HUVECs transfected with miR- 24-2 specific anti-miR as compared to HUVECs treated with N-Exo. The endothelial marker (VE-cadherin) ($p < 0.001$) showed a similar trend in expression levels as miR-24-2. However, VE-cadherin levels significantly increased ($p < 0.001$) in R-Exo-treated HUVECs, transfected with miR-24-2 specific miRs and elevation in the expression levels of mesenchymal marker (SMA-α) and TGF-β (indicative of fibrosis) takes place in R-exo-treated-HUVECs transfected with miR-24-2 specific miRs as compared to HUVECs treated with R-Exo (Fig 7B), thereby suggesting that EndoMT takes place in exosomes treated HUVECs.

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 (Fig 7C), thereby suggesting that EndoMT occurs in ECs lining the mitral valves of the heart and consequently leads to mitral valve fibrosis during RHD.

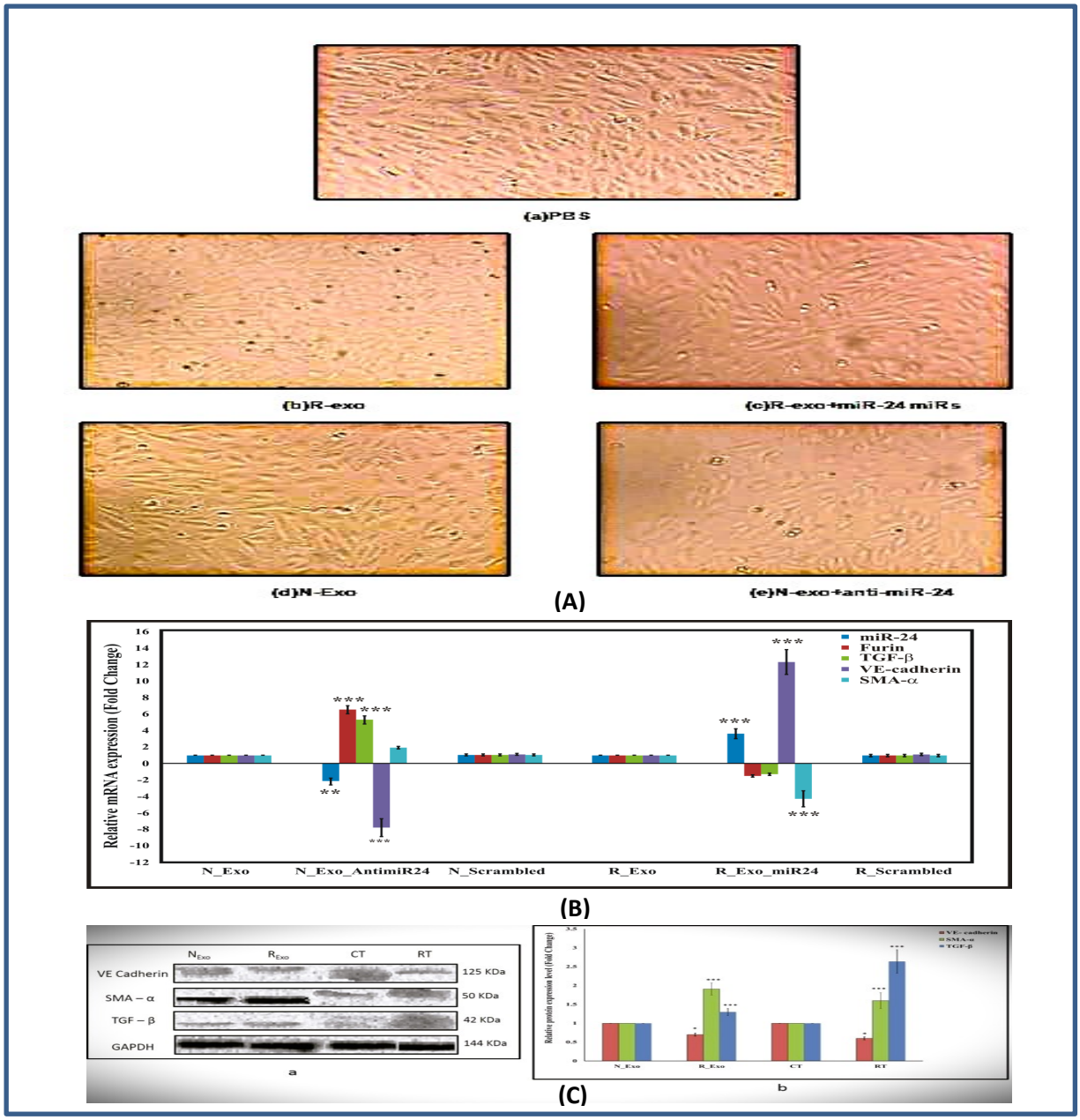


Fig 7 (A)Effect of treatment of miR-24-2-specific- miRs and anti-miRs in exosomes-treated HUVECs: Representative micrograph of HUVECs treated by (a) scrambled miR + PBS, (b) R-Exo, (c) R-Exo + miR- 24-2-miRs, (d) N-Exo, and (e) N-Exo + miR-24-2 anti-miRs. **(B.) Gene expression levels of exosomes treated HUVECs after transfection with miR-24-2 specific-miRs and anti-miRs:** 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 N-exo + miR-24-2 anti-miRs, as compared to respective controls. **(C) 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 normalized to GAPDH. (10X). Scale bar = 400 µm. All experiments were repeated three times. Each bar represents the mean ± S.D. Statistical analysis was carried out by one-way ANOVA. *$p < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control.

Haematoxylin-Eosin (H & E) and Mason trichrome (MT) staining of mitral valve tissues:
Haematoxylin-Eosin and mason trichrome staining of mitral valve tissues was carried out, which showed hyalinization (indicative of fibrosis) of the mitral valve tissues obtained from RHD patients as compared to the control tissues (Fig 8), thereby indicating that mitral valve fibrosis occurs during RHD.

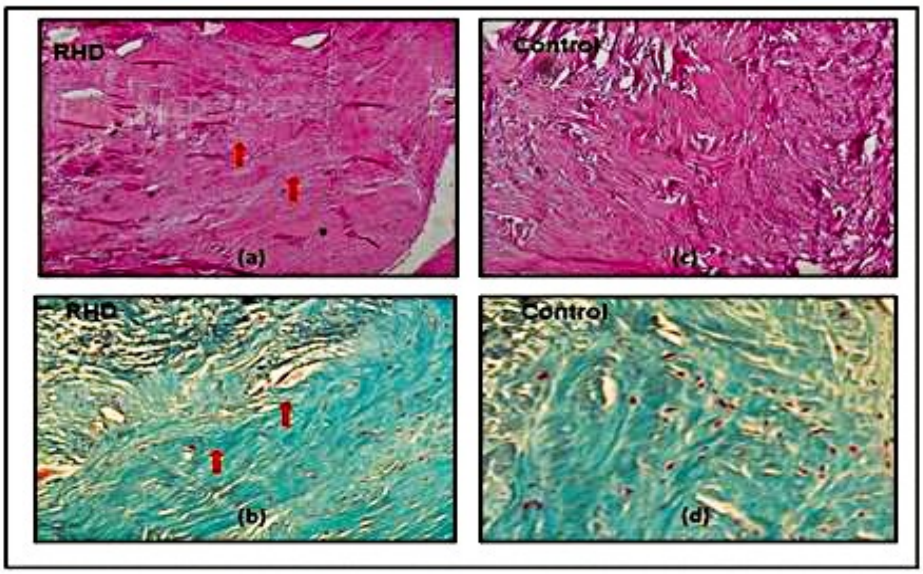


Fig 8 Haematoxylin-eosin and mason trichrome (HE and MT) staining of mitral valve tissues: Representative images of (a,c) HE and (b,d) MT-stained mitral valve tissues from RHD patients as compared to controls, respectively showing hyalinization in the tissues

Discussion

Rheumatic heart disease is an autoimmune disease that leads to permanent mitral valves damage. The mitral valves are lined by ECs, and these ECs undergo endothelial to mesenchymal transition. The mesenchymal cells are fibroblasts, and during mechanical injury or stressed/diseased conditions, 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]**. Various studies have been conducted that investigated the underlying causes for RHD pathogenesis. Till now, the exact pathogenesis of RHD remains obscure due to which there is no molecular diagnostic test and appropriate vaccine available. The existing literature focused on the genetic and epigenetic changes in various inflammatory and cell adhesion molecules, associated with RHD **[15].** Recently, emerging evidences suggest a vital role of exosomes in various cardiovascular diseases **[5].** Herein, the role of exosomes has been investigated in mitral valve fibrosis and dysfunction during RHD. In the present study, 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, and mesenchymal cells (myofibroblasts) are formed and these cells mostly express significantly increased expression levels of mesenchymal cell markers (SMA-α) as compared to endothelial cell markers (VE-cadherin). It was important to understand whether the miRNA content of exosomes is responsible for the mitral valve dysfunction during RHD. Interestingly, various studies have suggested the role of miR-24-2 during cardiac fibrosis **[12,13]**. Thus, exosomal miRNA sequencing analysis was done to reveal the de- regulated miRNA in all the biological samples, including the media of RHD-in vitro model. MiR-24-2 (down-regulated exosomal miR) was screened as a common miRNA in serum and PF, and media of the RHD *in- vitro* model. The ECs line the mitral valves, which are the sites where autoantibodies bind after an autoimmune response is generated; therefore, HUVECs were isolated and stimulated with pooled RHD patients' serum to generate an RHD *in-vitro* model in order to mimic the RHD *in-vivo* conditions in ECs. So, the significant down-regulated expression of exosomal miR-24-2 was found in exosomes of biological fluids (serum, PF)-*patient samples*, which can be correlated with RHD *in-vitro* model conditions attained via, media of RHD *in-vitro* model, which also showed the significantly decreased expression of exosomal miR-24-2. The expression of exosomal miR 24-2 was even found to be significantly reduced in R-Exo-treated HUVECs and mitral valve tissues of RHD patients.

The present study was mainly focused on the evaluation of gene expression responsible for causing mitral valve fibrosis. Therefore, miR-24-2 target gene, furin (TGF-β activator), and downstream TGF-β gene expression levels (responsible for cardiac fibrosis) was estimated in the mitral valve tissues, where it was observed that the expression levels of furin and TGF-β was significantly elevated. Finally, functional inhibition of miR-24-2 was performed in exosome-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 was checked in the mitral valve tissues of RHD patients, which also showed altered protein levels of these markers, suggesting the role of exosomal miR-24-2 in EndoMT and fibrosis, which can be correlated with EndoMT and fibrosis, taking place in ECs lining the mitral valves of the heart, eventually leading to mitral valve dysfunction. Though, the present study laid emphasis on the miRNA de-regulation in the patient samples, which can be one of the cause for RHD at genetic level, still the study needs to be done in a clinically focussed manner on a larger population size, to validate the findings of the study. Moreover, the development of RHD-*in-vitro* model was an advance and a challenging step to investigate the deeper and comprehensive understanding of this rare disease, still the study needs to be conducted on animal models to have even better understanding of the underlying pathogenic mechanisms during RHD. The collection of mitral valve samples from cadevers, requires profound knowledge and precise tissue handling techniques. Nonetheless, the present study, opens door for the deeper studies that can aid in finding the molecular theranostic tools for early detection and treatment during RHD.

To sum up, the exosomes in the biological fluids (serum and PF) are taken up by mitral valve ECs 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 target cells (ECs 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 lead to an increase in expression levels of its target gene, furin, which in turn, activates its downstream gene, TGF-β, expression levels, leading to mitral valve fibrosis.

Conclusion

In conclusion, in the present study, the role of exosomal miR-24-2 in causing mitral valve fibrosis and dysfunction during RHD pathogenesis has been investigated; where decline in expression levels of exosomal miR-24-2 is associated with increased severity during RHD. This study, therefore, can be used as a basal level study to check the role of other exosomal biomolecules such as proteins and lipids to better understand the cryptic RHD pathogenesis.

Availability of data and material

Yes

CRediT authorship contribution statement

Anuradha Chakraborti conceived the idea of the study. Shruti Sharma designed the framework, performed experimental work, and drafted the manuscript. Harkant Singh, Seema Chopra, and Uma Nahar provided the samples for the study and gave their critical comments. All authors approved the final version of the manuscript.

Code availability

None

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Compliance with ethical standards

Yes

Declaration of competing interest

The authors have nothing to disclose.

Ethics approval

Approval for the study was granted by the Institutional Ethics Committee, Postgraduate Institute of Medical Education and Research, Chandigarh (INT/IEC/2017/1457; Date: 04.12.2017).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Yes

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References

[1] Liu M, Lu L, Sun R, Zheng Y, Zhang P. Rheumatic heart disease: Causes, symptoms, and treatments. Cell Biochem Biophys 2015; 72:861–863.

[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; 183:507–511.

[3] Porter K, Turner NA. Cardiac fibroblasts: At the heart of myocardial remodelling. Pharmacol. Ther 2009; 123:255–278.

[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;314:928–939.

[6]Simons M, Raposo G. Exosomes: Vesicular carriers for intercellular communication, Curr. Opin. Cell Biol 2009; 21:575–581.

[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; 47:1286–1292.

[8] Ha M, Kim VN. Regulation of microRNA biogenesis, Nat. Rev. Mol. Cell Biol 2014; 15:509–524.

[9] Broughton JP, Lovci MT, Huang JL, Yeo GW,. Pasquinelli AE. Pairing beyond the seed supports microRNA targeting specificity, Mol. Cell 2016; 64:320–333.

[10] Vasudevan S. Posttranscriptional upregulation by microRNAs. Wiley Interdiscip. Rev. RNA 2012; 3:311–330.

[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, Miyazono K. Regulation of TGF-β-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; 16:2150–2160.

[14] Baudin B, Bruneel A, Bosselut N, Vaubourdolle M. A protocol for isolation and culture of human umbilical vein endothelial cells. Nat. Protoc 2007; 2:481–485.

[15] Rastogi M, Sarkar S, Makol A, Singh S, 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.

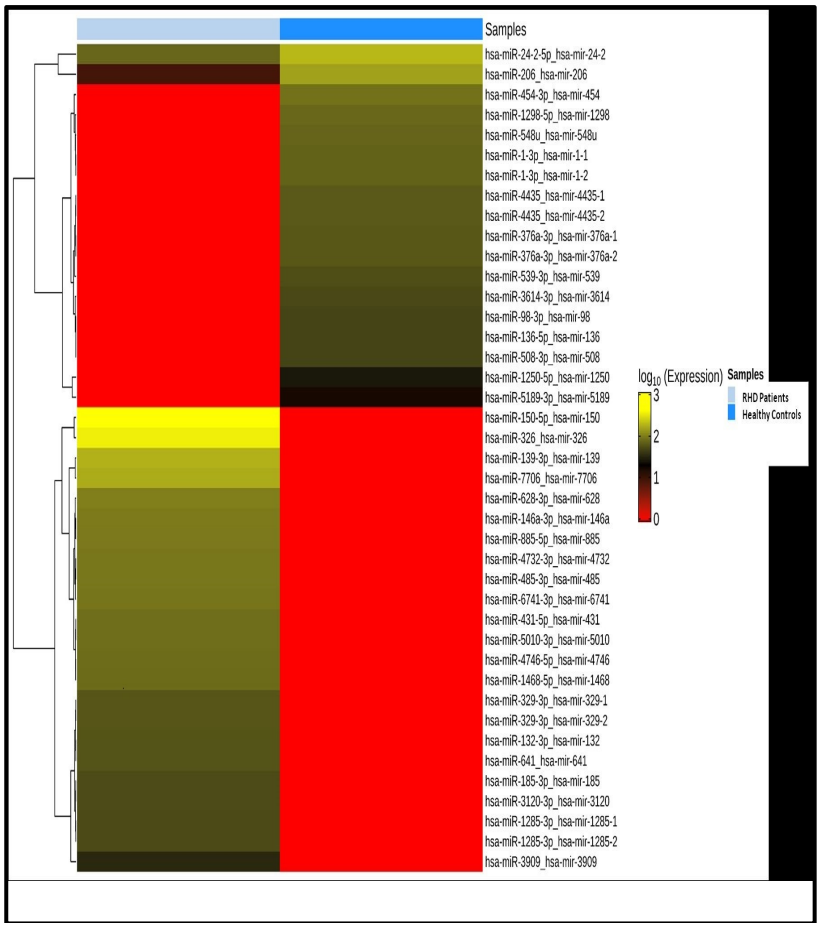
[16] Qu Q, Pang Y, Zhang C, Liu L, Bi Y. Exosomes derived from human umbilical cord mesenchymal stem cells inhibit vein graft intimal hyperplasia and accelerate reendothelialization by enhancing endothelial function. Stem Cell Res Ther 2010;11:133.

[17] Wong N, Wang. miRDB: An online resource for microRNA target prediction and functional annotations. Nucleic Acids Res 2015; 43:D146–D152.

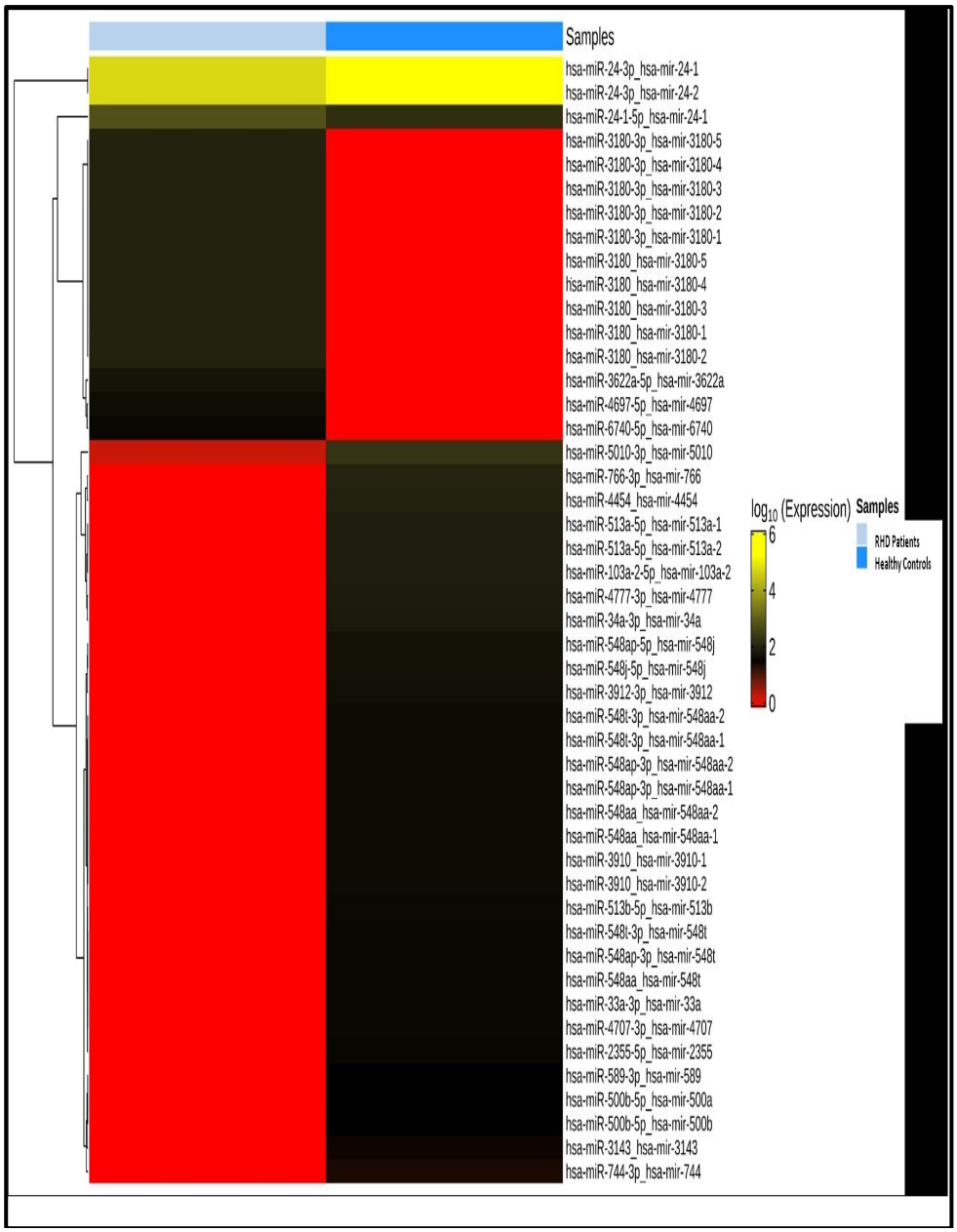
[18] Chen Z, Lu S, Xu M, Liu P, Ren R, Ma W. Role of miR-24, furin, and transforming growth factor-β1 signal pathway in fibrosis after cardiac infarction. Med. Sci. Monit 2017; 23:65–70.

[19] Sharma S, Sarkar S, Choudhury, Singh L, Singh H, Chakraborti A. Alpha-1-antitrypsin in serum exosomes and pericardial fluid exosomes is associated with severity of rheumatic heart disease. Molecular and Cellular Biochemistry 2023; https://doi.org/10.1007/s11010-022-04595-x

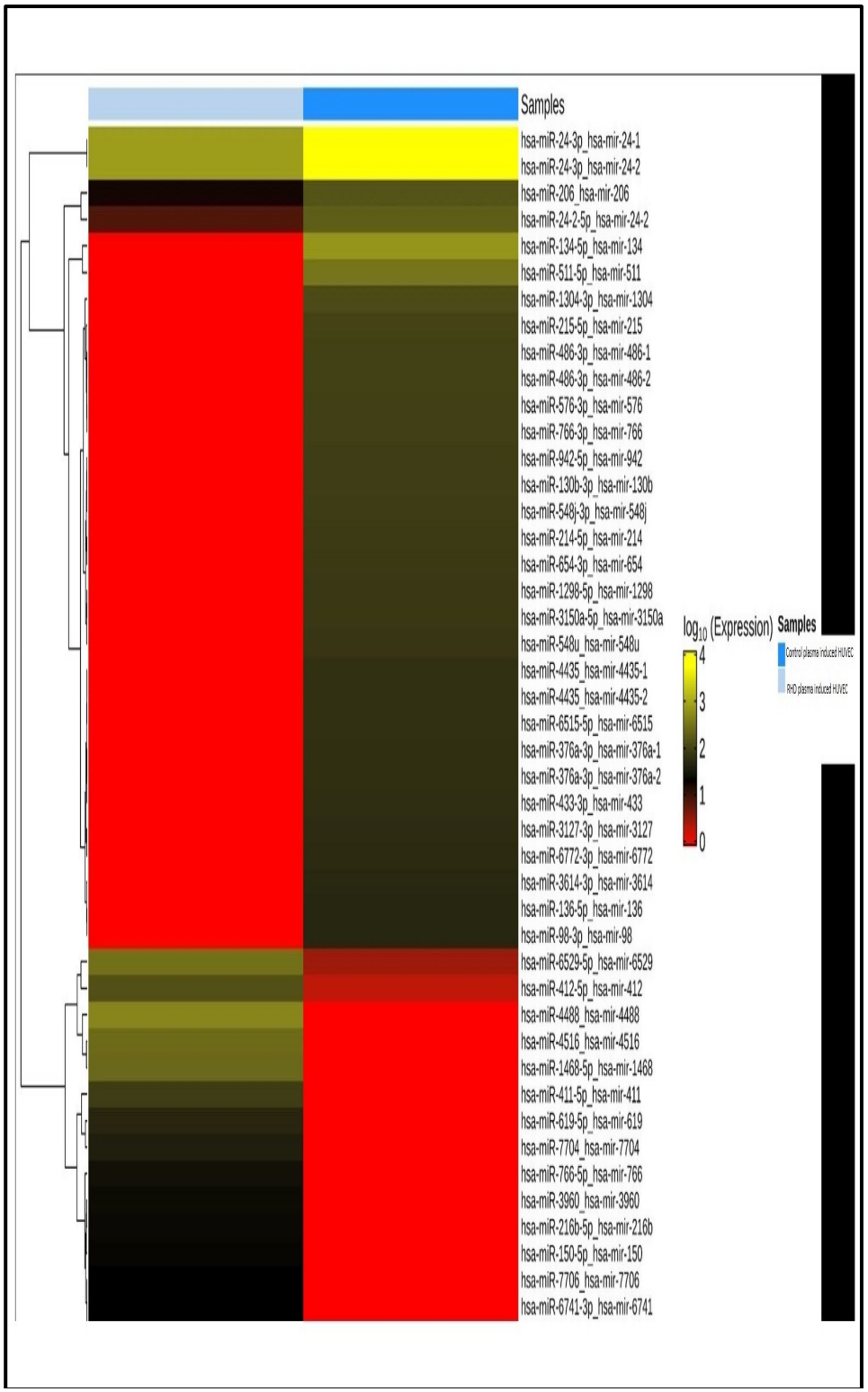
[20] Lu Q, Sun Y, Duan Y, Li B, Xia J, Yu S et al. Comprehensive microRNA profiling reveals potential augmentation of the IL1 pathway in rheumatic heart valve disease. BMC Cardiovasc. Disord 2018; 18:53.



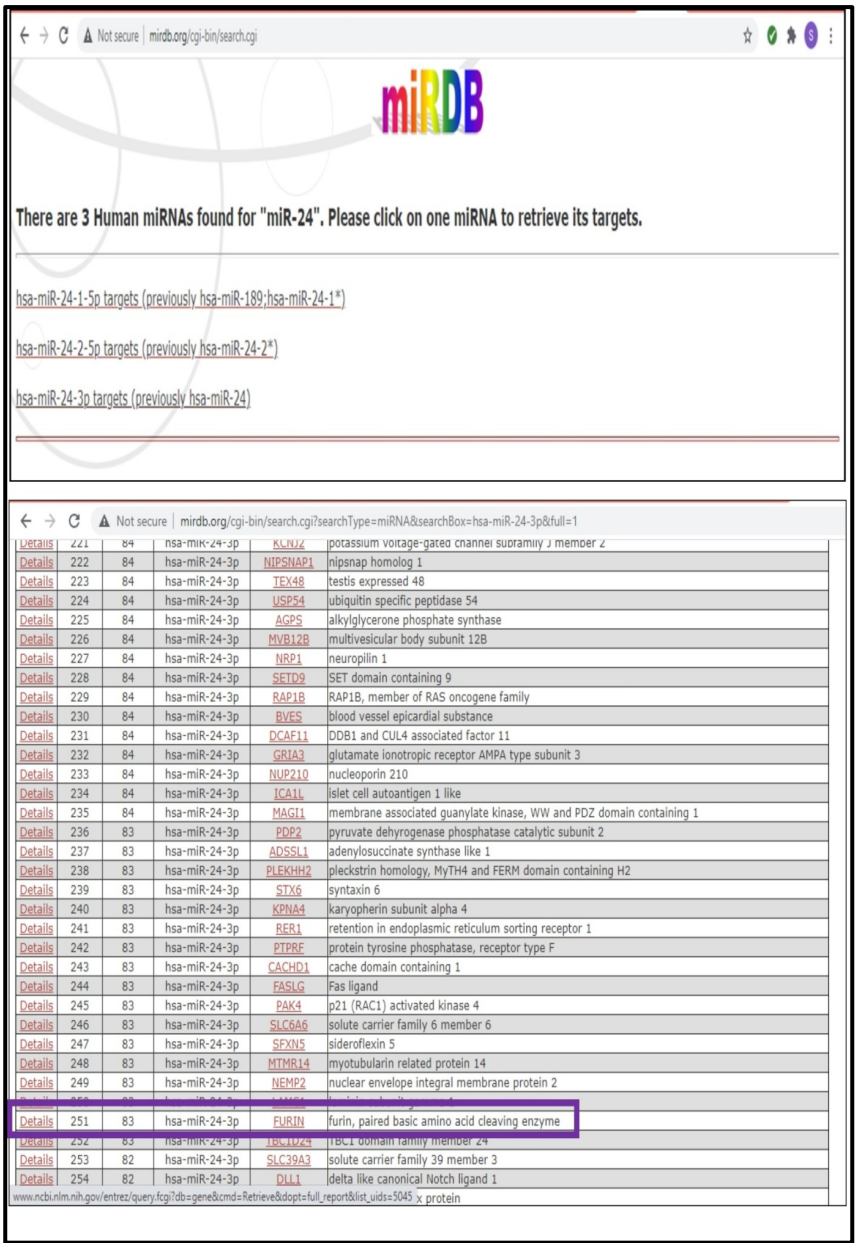
Supplementary Fig 1 Exosomal miRNA profiling in pooled RHD patients' serum showing differentially expressed miRNAs; yellow, upregulated miRNAs; grey, not differentially expressed miRNAs; red, downregulated miRNAs. The criteria required a minimum of twofold difference of log2 (fold change) in either direction.



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



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



Supplementary Fig MiR-24-2 target gene prediction using miRDB software analysis: A screenshot for miRDB target gene prediction analysis. Furin is one of the target genes highlighted in purple

Gene	Primer sequence :Forward (F) & Reverse (R)	Amplicon size
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

Table 1: Primers used in the study