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Sildenafil-mediated neovascularization and protection against myocardial ischaemia reperfusion injury in rats: role of VEGF/angiopoietin-1

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Abstract

Sildenafil citrate (SC), a drug for erectile dysfunction, is now emerging as a cardiopulmonary drug. Our study aimed to determine a novel role of sildenafil on cardioprotection through stimulating angiogenesis during ischaemia (I) reperfusion (R) at both capillary and arteriolar levels and to examine the role of vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) in this mechanistic effect. Rats were divided into: control sham (CS), sildenafil sham (SS), control + IR (CIR) and sildenafil + IR (SIR). Rats were given 0.7 mg/kg, (i.v) of SC or saline 30 min. before occlusion of left anterior descending artery followed by reperfusion (R). Sildenafil treatment increased capillary and arteriolar density followed by increased blood flow (2-fold) compared to control. Treatment with sildenafil demonstrated increased VEGF and Ang-1 mRNA after early reperfusion. PCR data were validated by Western blot analysis. Significant reduction in infarct size, cardiomyocyte and endothelial apoptosis were observed in SC-treated rats. Increased phosphorylation of Akt, eNOS and expression of anti-apoptotic protein Bcl-2, and thioredoxin, hemeoxygenase-1 were observed in SC-treated rats. Echocardiography demonstrated increased fractional shortening and ejection fraction following 45 days of reperfusion in the treatment group. Stress testing with dobutamine infusion and echocardiogram revealed increased contractile reserve in the treatment group. Our study demonstrated for the first time a strong additional therapeutic potential of sildenafil by up-regulating VEGF and Ang-1 system, probably by stimulating a cascade of events leading to neovascularization and conferring myocardial protection in *in vivo* I/R rat model.

Keywords: sildenafil • angiogenesis • VEGF • thioredoxin • blood flow

Introduction

In the field of cardiovascular research, a number of independent profiles or approaches have been explored to protect the heart from ischaemic damage. Current approaches to the treatment of ischaemic heart disease (such as coronary artery bypass graft surgery and angioplasty techniques) often results in incomplete revascularization because of the frequent presence of diffuse coronary artery disease extending into small peripheral vessels. As an alternative to these techniques, recent attention has been directed toward harnessing the body's ability to generate new blood vessels (natural angiogenesis). This report documents the results of animal studies that evaluated the ability of sildenafil, a highly selective inhibitor of phospho diesterase 5 (PDE-5) that recently has received considerable attention with respect to its cardioprotective and angiogenic potential, induce angiogenesis in myocardial infarction model. Sildenafil is the first drug developed for the treatment of erectile dysfunction in patients. Numerous experimental data in animals show that sildenafil has a preconditioning-like cardioprotective effect against ischaemia–reperfusion injury in the intact myocardium [1–4]. Sildenafil induces powerful cardioprotective effect *via* opening of mitochondrial K_{ATP} channel that helps to trigger the dilation of peripheral and coronary resistance arterioles. The other important effect of sildenafil is its nitric oxide driven capability that elicits the dilation of systemic veins and epicardial coronary arteries [3]. The impact of sildenafil in angina study, which included

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participants with left ventricular dysfunction, demonstrated a significant improvement in clinical outcome in patients with stable angina treated with sildenafil, suggesting a possible efficacy of this drug in treatment of heart failure [5]. Single-dose sildenafil was found to prolong exercise time in men with angina [5]. Previous findings support the notion that sildenafil may ameliorate heart failure associated with abnormal coronary microcirculation. Now it is well known that abnormalities of the microvasculature, such as a reduction in vascular density, thickening of the arteriolar wall and impairment of angiogenesis, contribute significantly to the pathogenesis of various forms of heart disease [6]. Thus, it is extremely important to develop the body's natural angiogenic process in order to create collateral circulation in areas where blocked coronary arteries deprive the heart muscle of sufficient blood flow, for example, in the settings of chronic myocardial ischaemia [7]. A modern experimental strategy for treating myocardial ischaemia is to induce neovascularization of the heart by use of '"angiogens', mediators that induce the formation of blood vessels, or angiogenesis [8, 9]. VEGF is a potent vascular endothelial cell specific mitogen that stimulates endothelial cell proliferation, microvascular permeability, vasodilatation and angiogenesis [10, 11]. VEGF is the only growth factor proven to be specific and critical for blood vessel formation [12–14]. Thus, VEGF plays a very important role in vascular formation whereas angiopoietins (Ang-1 and Ang-2) are known to stabilize vessel walls specially Ang-1.

Our prior studies have demonstrated that human coronary arteriolar endothelial cells (HCAEC) exposed to sildenafil (20 μM) significantly induce tubular morphogenesis with the induction of thioredoxin-1 (Trx-1, a redox molecule and plays an important role in angiogenesis), hemeoxygenase-1(HO-1) and VEGF [15].

Recent report from our laboratory demonstrated sildenafil-mediated expression of VEGF and Ang specific receptors such as kinase insert domain receptor (KDR), Tie-1 and Tie-2 [15]. Therefore based on the pre-existing data, we hypothesized that administration of sildenafil might induce myocardial angiogenesis through the stimulation of angiogenic factor/factors such as Trx-1, HO-1, VEGF/Ang-1 in the setting of acute ischaemia reperfusion model. This study constitutes the first report *in vivo,* which demonstrates sildenafil at a particular dose can (*i*) decrease endothelial cell injury after ischaemia and reperfusion, (*ii*) triggers angiogenic gene/protein expression, (*iii*) stimulates myocardial angiogenesis at both capillary and arteriolar levels (*iv*) increases blood flow, (*v*) improve left ventricular contractile functional reserve as demonstrated by stress test and (*vi*) increase fractional shortening and ejection fraction, as demonstrated by echocardiography.

Materials and methods

Endothelial cell culture

Human Umbilical Vein Endothelial Cells (HUVEC) was obtained from Lonza, Walkersville, MD and they were serially passaged. Cells were maintained in a culture medium, EGM-2 (Endothelial Growth Medium 2) supplemented with growth factors and antibiotics according to company specifications.

VEGF and Ang-1 siRNA treatment: The 20–25-nucleotide siRNAs were synthesized by Santa Cruz, CA, USA. VEGF siRNA (Cat# 29520) is a pool of 4-target-specific 20–25 nt siRNA desiged to knockdown gene expression of VEGF (corresponding to gene loc 1353 sense 5′-GAUUAUGCGGAUCAAACCUTT-3′ & antisense 5′-GGUUUGAUCCGCAU AAUCTT-3′, 499 sense 5′-AACACAGACTCGCGTTGCA-3′ & antisense 5′-TGCAA CGCGAGTCTGTGTT-3′, 359 sense 5′-TGAGCT TCCTACAGCACAA-3′ & antisense 5′-TGTGCTGTAGGAAGCTCA -3′, 538 sense 5′-AACGAACGTACTTGCAGAT-3′ & antisense 5′- TCTGCAAG TACGTTCGTT-3′, respectively). Ang-1 siRNA is a pool of 6-target-specific 20–25 nt siRNA desiged to knockdown gene expression of Ang-1 (cat#sc-37198) (corresponding to gene loc 1086 sense 5′-GGAAGAGUUGGACACCU UAtt-3′ and antisense 5′-UAAGGUGUCCA ACUCUUCCtt-3′, 1302 sense 5′GGAAGA GAAACCAUUUAGAtt-3′ & antisense 5′- UCUAAAUGGUUUCUCUUCCtt -3′, 807 sense 5′-CCACGAGACUUG-AACUUCAtt-3′ & antisense 5′-AAGUUCAAGUCUC-GUGGtt -3′, 1161 sense 5′-CCACUGUUGCUAAAGAAGAtt-3′ and antisense 5′-UCUUCUUUAGCAACAGUG Gtt-3′, 572 sense 5′-CUGAACCA GACAUCAAGAAtt-3′ and antisense 5′-UUCUUGAU GUC UGGUUCAGtt-3′, 1135 sense 5′-GAA-CUGG AAGGAUU ACAAAtt-3′ and antisense 5′-UUUGUA-AUCCUUCCAGU UCtt-3′ respectively). The cells were transfected with siRNA for VEGF and Ang-1 according to the company specifications. HUVECs were subcultured in 12-well tissue culture plate. The HUVECs were grown in medium without antibiotics at this stage as it is critical for successful transfections. The cells were incubated at 37°C in a CO_2 incubator. After attaining confleuncy, 60–70% of the cells were transfected with siRNA along with the transfection reagent and transfection medium. The cells were incubated for 72 hrs for complete transfection. The medium was aspirated and the cells were washed with Phosphate Buffered Saline (PBS). The cells were trypsinized, neutralized with trypsin-neutralizing solution and collected, counted, and 4×10^4 transfected cells were used for tuburogenesis.

Tube formation on Matrigel after sildenafil treatment in normal and siRNA (VEGF and Ang-1) transfected cells

Confluent normal HUVEC and siRNA (VEGF and Ang-1) transfected HUVEC's were treated with different concentration of sildenafil (100 nM, 10 μM and 20 μM) for tube formation. In brief, 250 μl of ice cold Matrigel (BD Bioscience, Bedford, MA, USA) was coated on a 24-well cell culture plate as a base for tube formation. After allowing the gel to settle for 30 min. in a 37°C, 5% CO_2 incubator, the endothelial cells (4×10^4) from normal and siRNA pre-transfected cells were seeded onto the Matrigel with sildenafil and incubated overnight at 37°C, in a CO_2 incubator. After 18 hrs exposure to sildenafil, the extent of tube formation was recorded by the phase-contrast microscope (magnification × 200) with a digital camera.

Animals

This study was performed in accordance with the principles of laboratory animal care formulated by the National Society for Medical Research and with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (Publication No. 85–23, revised 1985). The experimental protocol

was approved by the Institutional Animal Care Committee of the Connecticut Health Center (Farmington, CT, USA).

Experimental design and surgical procedure

Male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) weighing 250–275 g were used in this study. Rats were divided into four groups: Control sham (CS), sildenafil sham (SS), Control + IR (CIR) and sildenafil +IR (SIR). Sildenafil (Viagra - 50 mg) tablets were dissolved in saline. Rat were anaesthetized and mechanically ventilated. Bolus of sildenafil (0.7 mg/kg. body wt ([16, 17]) was given through the tail vein 30 min. before ischaemia. This dose compares well with the dose in humans (50 mg/70 kg) [18]. The surgical procedure was carried out as described previously [19, 20]. A 6-0 polypropylene suture was passed under the left anterior descending coronary artery (LAD) at the level of the left atrial appendage. A 10-mm section of polyethylene tube was placed on top of the LAD to secure the occlusion without damaging the artery. Both ends of the suture were passed through a segment of flared PE160 tubing to form a snare. Ischaemia was induced by pulling the snare and clamping tube with a hemostat for 30 min. Myocardial ischaemia was confirmed visually *in situ* by regional cyanosis, ST elevation and depression, or T wave inversion on the electrocardiogram, and hypokinetic or dyskinetic movement of the myocardium. After 30 min. of ischaemia, the snare was released, and the heart was allowed to reperfuse for 1, 2, 4 and 7 days depending on the protocol. Reperfusion was readily confirmed by hyperaemia over the surface of the previously ischaemic-cyanotic segment. After completion of all surgical protocols, the chest wall was closed in layers, as described previously [20]. After surgery, analgesic buprenorphine (0.1 mg/kg s.c.) was given and the animals were weaned from the respirator, and were then placed on a heating pad while recovering from anaesthesia. ECG was monitored during the protocol of ischemia and reperfusion.

Haemodynamics

After surgical procedures, the rats were anaesthetized and ventilated. The arterial catheter was advanced into the left ventricle through the right carotid artery. Myocardial function was assessed as the maximum values of the first derivative of left ventricular pressure ($LVdp/dt_{max}$) using PowerLab (ADInstruments, Colorado Springs, CO, USA) with and without intravenous administration of dobutamine (5 μg/kg per min.), as previously described [19].

Regional myocardial blood flow

Regional myocardial blood flow, 14 days after ischaemia, was measured with stable gold-labelled microspheres (BioPAL, Worcester, MA, USA) as previously described. Approximately 1,000,000 gold-labelled microspheres were injected into the left ventricle. Simultaneously, a reference blood sample was withdrawn (0.5 ml/min.) from right femoral artery through an arterial catheter. After sampling the blood, the animals were euthanized with an intravenous injection of KCl. The heart was excised and the risk area of left ventricular tissue was carefully dissected under the stereomicroscope and weighed. All tissue and blood samples were sent to BioPAL and the number of microspheres was counted with spectrophotometric analysis. Myocardial blood flow (ml/min/g tissue) was calculated. Blood flow (tissue sample X; ml min^{-1} g^{-1}) = [withdrawal rate (ml/min)/ weight (tissue sample X; g)] × [γ counts (tissue sample X)/ counts (reference blood sample)]. Samples with significant infarct (>50% of tissue sample) were excluded from regional blood flow analysis [20].

Measurement of infarct size

Animals ($n = 6$) were anaesthetized 24 hrs after ischemia and mechanically ventilated, chest wall was opened as described above. The suture left around the LAD was identified and ligated permanently. Unisperse blue (50%) was injected through right jugular vein and infarct size and area of risk were measured in the eight horizontal sections between the point of ligation and the apex as previously described [21]. The area at risk was recognized as the area not perfused with 50% Unisperse blue (Ciba-Geigy, Glen Ellyn, IL, USA), whereas the non-infarcted and infarcted areas were demarcated after incubation with 1% triphenyl tetrazolium chloride (Sigma, St. Louis, MO, USA). With the use of Scion image software (Scion Corporation, Frederick, MD, USA), the volumes of both infarcted myocardium and area at risk were calculated. Infarct size was reported as a percentage of the area at risk.

Determination of cardiomyocyte and endothelial cell apoptosis

The rat hearts were harvested at pre-determined times as per the protocol for paraffin-embedded or frozen tissue sectioning. Double-fluorescent immunohistochemical determination of cardiomyocyte apoptosis was performed with terminal dUTP nick end labelling (TUNEL) assay on deparafinized 4 μm thick sections using an *In Situ* Cell Death Detection Kit, fluorescein as per the kit protocol (Roche Diagnostics, Mannheim, Germany). Antibody against α-sarcomeric actin (Sigma, St. Louis, MO, USA) was used for cardiomyocyte apoptosis [22, 23] and von-willebrand factor for endothelial cell apoptosis [20]. The number of TUNEL-positive cardiomyocytes was counted on 100 high power fields.

Quantitative real-time RT-PCR

Total RNA was isolated from left ventricular tissue using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer. The risk area was used for the isolation of total RNA. Reverse transcription proceeded with 1 μg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative real-time RT-PCR was carried out with iCycler iQ (Bio-Rad Laboratories). The level of transcripts was shown as a relative expression level using β-actin transcripts as a standard. The primer sets used in quantitative real-time RT-PCR for VEGF was forward, 5′-CAGGAGTACCCCGATGAGATAG-3′ reverse, 5′-GATCT GCATAGTGACGTTGCTC-3′, for Ang-1 was forward, 5′-GTGAGAGTGCGAC AGAGCAG-3′ reverse 5′-CAAGTTTTTGCAGCCACTGA-3′ for Ang-2 was forward, 5′-GGCTCAGAGGTCATGAGGAG-3′ reverse 5′-AAACCATGGCACTTCTGTCC-3′.

Western blot analysis

The risk area from the left ventricle was isolated and used for Western blot analysis. To quantify the abundance of the angiogenic (VEGF, Ang-1), redox

regulatory (Trx-1, HO-1) and anti-apoptotic protein (Bcl-2) following 2, 4 and 7 days after reperfusion, we performed standard Western blot using specific antibodies (Santa Cruz, CA & Cell Signaling, MA, USA) as per the company specifications [19]. Similarly, Western blotting was also performed for p-Akt and p-eNOS using specific antibodies (Cell Signaling, MA, USA).

Measurement of capillary and arteriolar density

The risk area from the left ventricle was used for the measurement of capillary and arteriolar density. For the measurement of capillary density, endothelial cells were labelled using mouse monoclonal anti-CD31/PECAM-1 (1:100, Pharmingen, San Diego, CA, USA) followed by a biotinylated horse antimouse secondary antibody (1:200 dilution). The reaction product (brown) was visualized with Diamino Benzidine (DAB) substrate using the Vector ABC Vectastain Elite Kit (Vectorlabs, Burminghame, CA, USA). On separate slides, for arteriolar density measurement vascular smooth muscle cells were labelled using mouse monoclonal anti-smooth muscle actin (1:50, Biogenex, San Ramon, CA, USA) followed by a biotinylated rat-adsorbed horse antimouse secondary antibody (1: 200 dilution). The reaction product (violet) was visualized with VIP substrate using the Vector ABC Vectastain Elite Kit (Vectorlabs). Images were captured and stored in digital tiff file format for later image analysis. Counts of capillary density and arteriolar density per mm^2 were obtained after superimposing a calibrated morphometric grid on each digital image using Adobe Photoshop Software.

Echocardiography

Each rat was sedated using isoflurane (3%, inhaled). When adequately sedated, the rat was secured with tape in the supine position in a custom-built mould designed to maintain the rat's natural body shape after fixation. The animal's paws were anchored to the electrodes on the platform to obtain electrocardiography, respiratory rate and heart rate and body temperature was maintained at 37°C throughout the procedure. The hair on the chest wall was removed with a chemical hair remover. Ultrasound gel was spread over the precordial region and the ultrasound biomicroscopy (UBM). (Vevo 770, Visual-Sonics Inc., Toronto, Canada) with a 25-MHz transducer was used to visualize the left ventricle. Left ventricle was analysed in apical, parasternal long axis and parasternal short axis views for left ventricular systolic function, left ventricular cavity diameter, wall thickness and diastolic function determination. Myocardial infracted segment was determined according to its kinetics: hypokinetic (reduction in wall thickness), akinesis (no wall motion) and dyskinesis (unsynchronized movement of segment with normal myocardium). 2D-directed M-mode image of the left ventricular short axis were taken just below the level of the papillary muscles for analysing ventricular wall thickness and chamber diameter. All left ventricular parameters were measured according to the modified American Society of Echocardiography-recommended guidelines. Ejection fraction (EF) and fractional shortening (FS) were assessed for left ventricular systolic function. Diastolic function was assessed in apical four chamber view, by measuring mitral peak flow velocity of the E-wave and A-wave in centimetres per second (cm/s) and the ratio between these two waves (E/A ratio). We have also measured the deceleration time (DT) which signifies the chamber stiffness [24, 25]. All measurements represent the mean of at least three consecutive cardiac cycles.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Differences between groups were tested for statistical significance by Student's t-test ($P < 0.05$).

Results

Tubular morphogenesis by HUVECs after sildenafil treatment with and without VEGF/Angiopoietin-1 siRNA

Earlier we have shown that sildenafil treatment form tube-like structures in a dose-dependent manner [15]. The formation of capillary network of tubular structure was extremely prominent when the cells were exposed to 10 μM and 20 μM sildenafil. In separate experiments the cells were treated with sildenafil in HUVECs which were pre-transfected with siRNA for VEGF and Ang-1 to demonstrate sildenafil-mediated tuborogenesis involves VEGF and Ang-1. As expected, cells pre-treated with VEGF and Ang-1 siRNA have demonstrated inhibition of sildenafil-mediated tubular morphogenesis of HUVEC which was observed in corresponding control sildenafil-treated cells (Fig. 1).

Effect of sildenafil on infarct size, contractile reserve and blood flow

Myocardial infarct size was measured following 24 hrs of reperfusion to examine whether sildenafil treatment reduces the cardiac tissue damage following ischaemia /reperfusion. Infarct size, expressed as percent of infracted to total area at risk, was noticeably decreased to 20% in the SIR group compared to the CIR group (39%) (Fig. 2A). Pharmacological cardiac stress testing with dobutamine infusion revealed difference in the extent of cardiac contractile reserve between the treated and non-treated groups. The sildenafil-treated group SIR displayed significantly elevated contractile reserve as judged from the improved dp/dt_{max} (Fig. 2B). Measurement of the regional blood flow with stable gold-labelled microspheres technique revealed that sildenafil treatment increased blood flow by 2-fold as compared with controls following 14 days of reperfusion (Fig. 2C).

Effect of sildenafil on cardiomyocyte and endothelial cell apoptosis

Double antibody staining with anti-α-sarcomeric actin and anti-von Willebrand factor by TUNEL assay was used to measure the

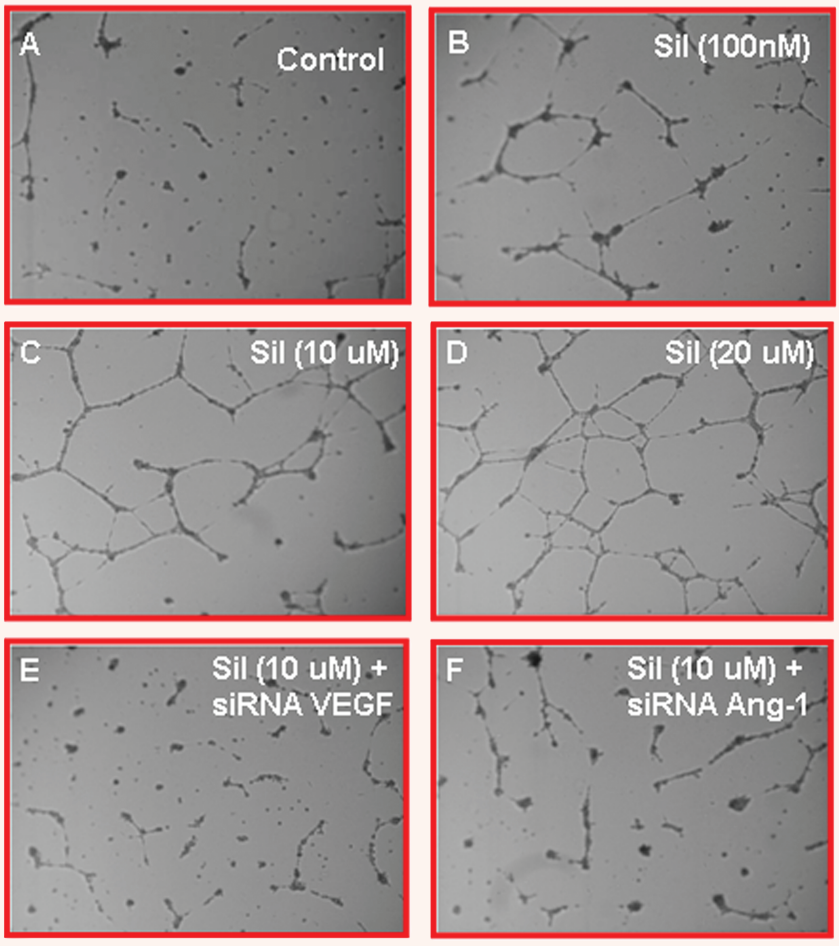

Fig. 1 Sildenafil treatment induced tube-like structure. Human umbilical vein endothelial cells (HUVECs) were exposed to various concentration of sildenafil ranging from 100 nM (Fig. 1B), 10 μM (Fig. 1C), 20 μM (Fig. 1D). The tube formation was very prominent when cells were exposed to 10–20 μM. VEGF siRNA (Fig. 1E) and Angiopoietin-1 siRNA (Fig. 1F) treated HUVECs abolished the tube formation induced by sildenafil.

cardiomyocyte (Fig. 3A and B) and endothelial cell apoptosis (Fig. 3C and D). Significant increase in the number of TUNEL-positive cells was observed in the CIR group. This effect was strongly inhibited by sildenafil in the SIR group. Decreased cardiomyocyte apoptosis was observed in sildenafil-treated rats as compared to controls following 24 (304 *versus* 56) and 48 (168 *versus* 16) hrs of reperfusion. Similarly, reduced endothelial apoptosis was observed after 24 (243 *versus* 100) and 48 hrs (167 *versus* 76) of reperfusion in the SIR group as compared to CIR group.

Effect of sildenafil on capillary density and arteriolar density

Immuno-histochemical staining of PECAM-1 was performed to observe the capillary density (CD-31). At 400× magnification, eight non-overlapping random fields, each selected from non-infarcted risk area were used for CD-31 counting. Four sections from each heart were examined. The capillary density in the peri-infarct area was significantly greater in the sildenafil-treated group (4617 *versus* 3298 and 4978 *versus* 3449 counts/mm^2) than in the CIR group following 2 & 4 day after reperfusion (Fig. 3E and F). Sildenafil-treated group documented significant increase in arteriolar density (Fig. 3G and H) following 7 days of reperfusion as compared to the CIR group (5.40 *versus* 2.16).

Effect of sildenafil on the mRNA expression of VEGF, Ang-1, Ang-2 by real-time RT-PCR

Increased expression of VEGF (1.9-, 2- and 4-fold) and Ang-1 (5-, 11- and 23-fold) mRNA was observed on treatment with sildenafil

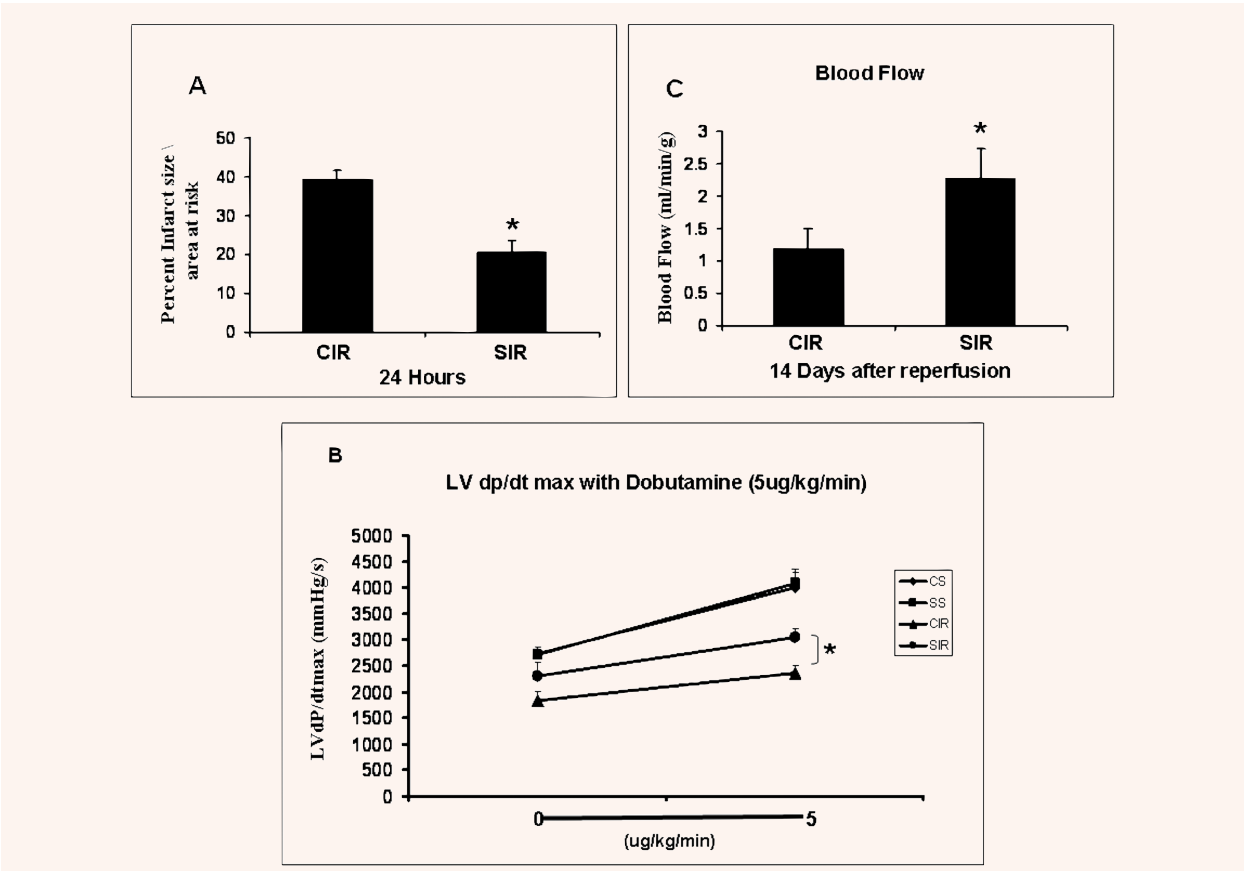

Fig. 2 Effects of sildenafil on infarct size, dp/dtmax and blood flow. (**A**) Bar graph shows infarct size measured 24 hrs after reperfusion. (**B**) Measurement of dp/dt_{max} under dobutamine stress test. Changes in dp/dt_{max} in mm of mercury/s (mmHg/s) from baseline (0 μg/kg/min) to a higher bolus dose of dobutamine (5 μg/kg/min) in 14 days I/R hearts; (**C**) Regional blood flow of risk area was measured with gold label neutron activation technique. Blood flow was estimated 14 days after reperfusion. Values are mean ± S.E.M. ($N = 6$). *$P < 0.05$ compared with control. CIR represents control IR and SIR represents sildenafil IR group.

8, 12 and 24 hrs after reperfusion as compared to the control (Fig. 4A and B). In contrast, significant increase was observed in Ang-2 level following 8 hrs of reperfusion which was reduced 12 and 24 hrs after reperfusion (Fig. 4C).

Effect of sildenafil on the expression of survival proteins (p-eNOS, p-Akt)

The reduction of cardiomyocyte and endothelial cell apoptosis point towards the critical involvement of survival proteins, such as eNOS and AKT in the sildenafil-induced cardioprotection. We quantified the expression of these proteins and their phosphorylation status. The non-phosphorylated Akt and eNOS were used as the loading controls. As expected, we observed increased phosphorylation/activation of Akt (2-fold) and eNOS (4.5-fold) in the sildenafil-treated group as compared to the control following 8 hrs of reperfusion (Fig. 5A–D).

Effect of sildenafil on the expression of the redox regulatory (HO-1, Trx-1), angiogenic (VEGF, Ang-1) and anti-apoptotic (Bcl-2) proteins

We examined the expression of these proteins in rat's left ventricular tissue following 2, 4 and 7 days of reperfusion. We examined the levels of the stress-related protein HO-1 which is a downstream regulator of Trx-1 as per our previous findings [15]. Expression of HO-1 (Fig. 6A) was found to be increased by 2.8-, 4- and 1.6-fold following 2, 4 and 7 days of reperfusion respectively in the sildenafil administered group as compared to the control. The Trx-1 level was found to be increased by 1.6-, 1.2- and

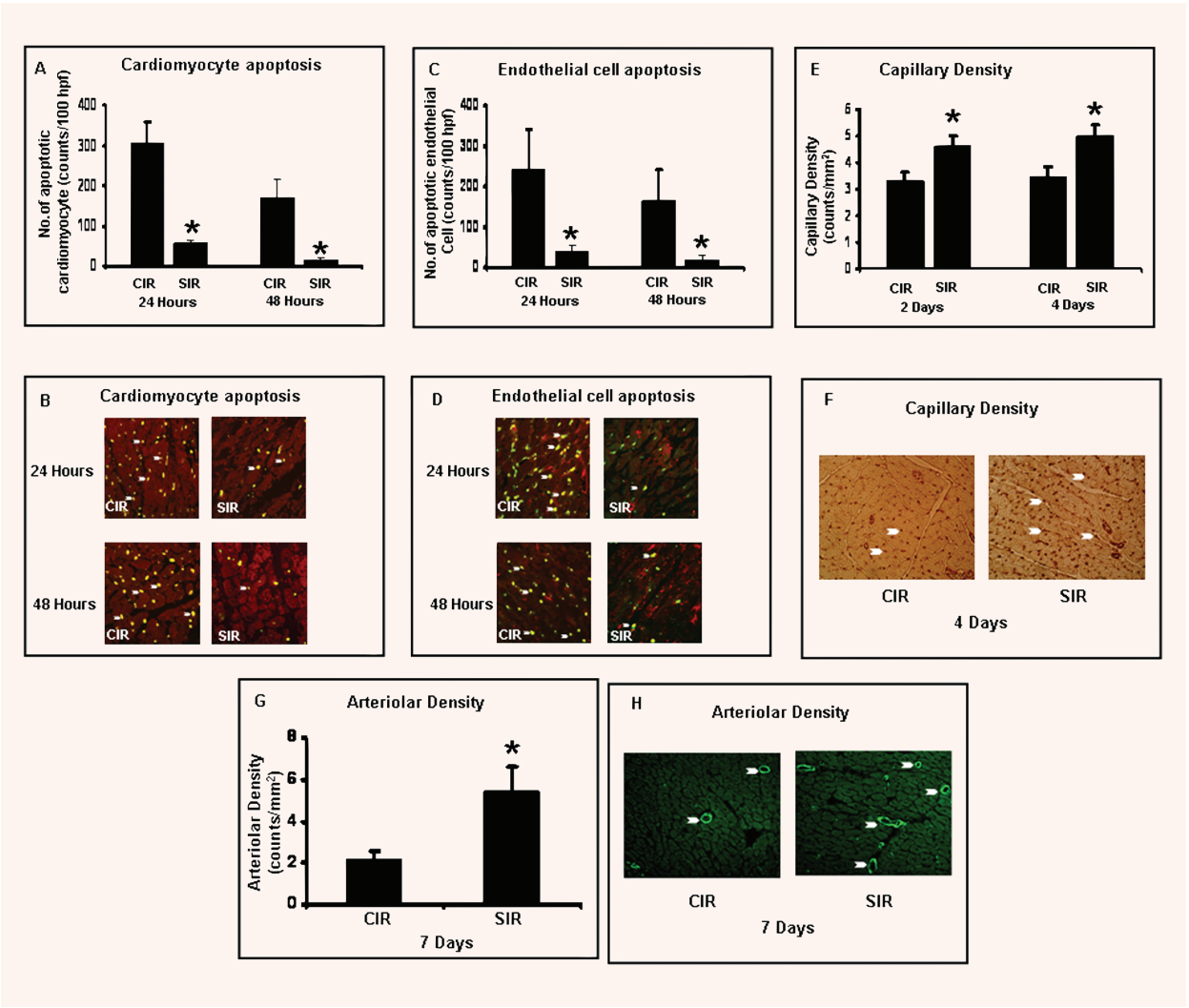

Fig. 3 Effect of sildenafil on apoptosis, capillary density and arteriolar density. (**A**) Cardiomyocyte apoptosis following 24 and 48 hrs of reperfusion. (**B**) shows representative pictures for cardiomyocyte apoptosis in green fluorescent colour denoted by white arrows (**C**) Endothelial apoptosis following 24 and 48 hrs of reperfusion. (**D**) Shows representative pictures for endothelial apoptosis in green fluorescent colour denoted by white arrows. (**E**) Left ventricular endocardial capillary density following 2 and 4 days of reperfusion. Endothelial cells were labelled using mouse monoclonal anti-CD31/PECAM-1 for capillary staining and eight non-overlapping random fields were selected from endocardial regions on non-infarcted area of the left ventricle (two sections from each heart); (**F**) Shows the representative pictures for capillaries stained in brown by DAB and denoted by white arrows following 4 days of reperfusion; (**G**) Left ventricular endocardial arteriolar density following 7 days of reperfusion. Tissue sections were labelled using monoclonal anti-smooth muscle actin and eight non-overlapping random fields were selected from endocardial region of the left ventricle; (**H**) Representative pictures for arterioles stained in green fluorescence and denoted by white arrows following 7 days of reperfusion. Values are mean ± S.E.M. ($N = 6$). $^*P < 0.05$ compared with CIR. CIR represents control IR and SIR represents sildenafil IR group.

1.5-fold following 2, 4 and 7 days of reperfusion respectively in the SIR group as compared to the CIR group (Fig. 6B).

The sildenafil-treated group exhibited 10- and 2.6-fold increase in VEGF expression as compared to their corresponding controls, following 2 and 4 days of reperfusion respectively (Fig. 6C). We also observed a 2.7-, 1.7- and 3-fold increase in Ang-1 expression as compared to their corresponding controls following 2, 4 and 7 days of reperfusion respectively (Fig. 6D).

We also observed increased expression of anti-apoptotic protein Bcl-2 (3.3-, 3.1- and 2.8-fold as compared to the corresponding controls following 2, 4 and 7 days of reperfusion, respectively) in SIR group (Fig. 6E). Protein quantification was done from six sim-

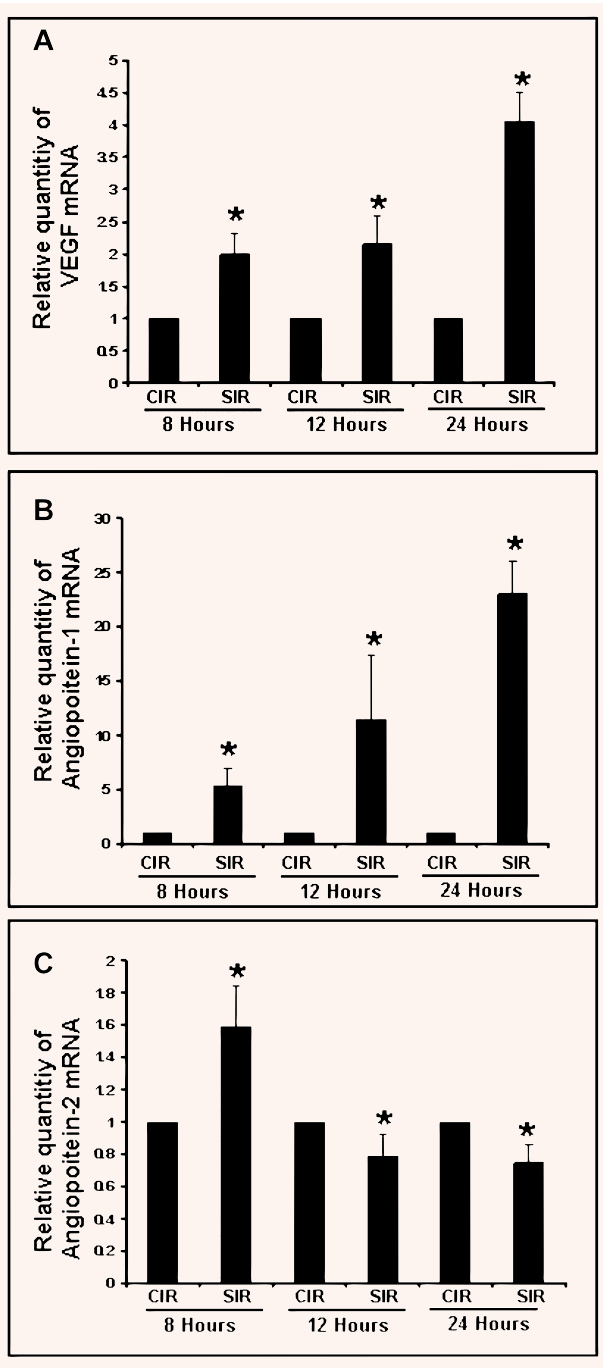

Fig. 4 Effect of sildenafil on VEGF, Ang-1 and And-2 mRNA levels. Bar graph represents quantitative measurement of Real Time RT-PCR analysis between control I/R (CIR) and Sildenafil I/R groups following 8, 12 & 24 hrs of reperfusion: (**A**) VEGF mRNA; (**B**) Angiopoietin-1 mRNA; (**C**) Angiopoietin-2 mRNA * $P < 0.05$ compared with CIR.

ilar experiments and GAPDH was used as the loading control for all proteins.

Echocardiography analysis

Echocardiography performed after 45 days of myocardial ischaemia has shown no significant difference between CS and SS groups, but SIR group showed significant increase in contractile ability of the peri-infarcted area suggesting the ability of sildenafil to improve the area at risk after myocardial ischaemia to recover substantially from further deterioration as observed in CIR animals. Thus, reduced chamber dilatation and increased left ventricular systolic function was observed resulting in improved myocardial remodelling in the treatment groups. EF of left ventricle was significantly increased in the SIR group compared to CIR group (62.27 $\pm$ 2.79 *versus* 53.65 $\pm$ 3.05). FS, a measure of systolic function, was also found to be increased significantly in the SIR group compared to CIR (35.23 $\pm$ 2.4 *versus* 29.19 $\pm$ 1.2). Left ventricular inner diameter (LVID) was significantly reduced in the SIR group compared to CIR suggesting reduced volume overload in SIR group (Fig. 7). Moreover, significant difference was noted in both LVAW (left ventricular arteriolar wall) and LVPW (left ventricular posterior) during systole in SIR group compared to CIR. Diastolic function as assessed by transmitral flow velocity, there was minimal diastolic dysfunction in SIR group with E/A ratio of 1.10 $\pm$ 0.02 compared to controls which were having pseudonormal pattern of filling with E/A of 3.61 $\pm$ 0.13 (Fig. 7). DT was shorter in the CIR group as compared to the SIR group showing increased chamber stiffness in the CIR group.

Discussion

The present study reports for the first time that intravenous administration of a clinically relevant dose of sildenafil (0.7 mg/kg), 30 min. prior to the LAD occlusion, followed by reperfusion resulted in the activation of VEGF/Ang-1 system both at the mRNA and protein level in the left ventricular myocardium leading to angiogenesis. The treatment with sildenafil also caused a marked increase in the myocardial capillary and arteriolar density, suggesting that sildenafil promotes the formation of functional coronary microvessels leading to increased blood flow in the failing heart. We found that sildenafil promote the induction of angiogenesis by triggering angiogenic regulatory proteins along with the phosphorylation of eNOS and Akt. Such an angiogenic effect of sildenafil *in vivo* may be a direct action. In this regard, we have recently reported that exposure to sildenafil significantly accelerates tuborogenesis in HCAEC on matrigel along with the activation of VEGF/Ang-1 system [15]. It is also known that VEGF and Ang-1 stimulate angiogenesis under both physiological and pathological conditions [26, 27]. In our experimental model, perhaps

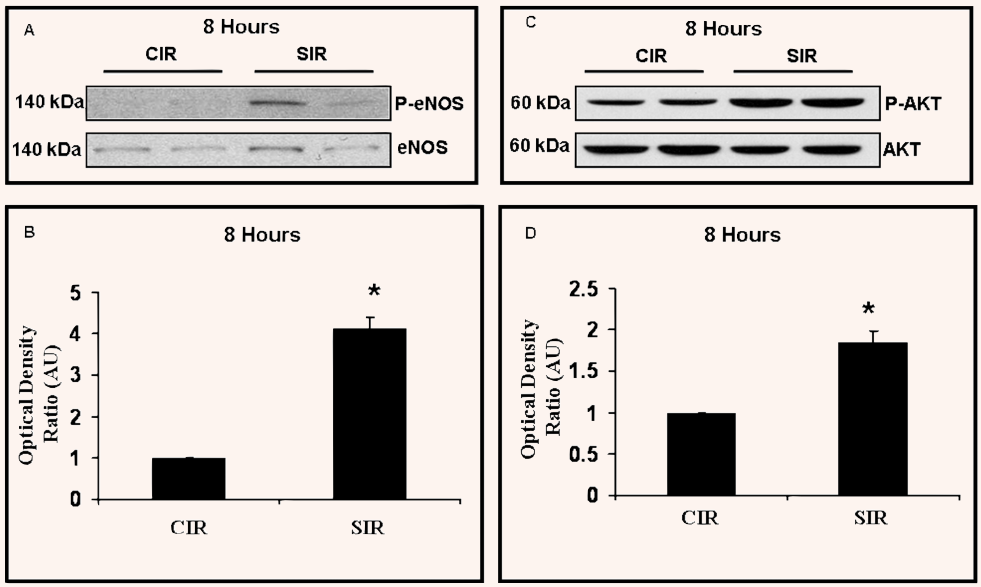

Fig. 5 Effect of sildenafil on phosphorylation of eNOS and Akt. **A** and **C** show a representative Western blot for the effects of sildenafil on the expression of phosphorylated-eNOS and phosphorylated-Akt in CIR and SIR following 8 hrs of reperfusion. p-eNOS and p-Akt were expressed as 140 and 60 kD respectively. eNOS and Akt were used as loading controls. Bar graphs in **B** and **D** represent the quantitative measurement in arbitrary units for p-eNOS and p-Akt respectively. $^{*}P < 0.05$ compared with CIR. CIR represents control IR group and SIR represents sildenafil IR group.

the most significant and interesting trend was the apparent relationship between Ang-1 and Ang-2 protein levels. Ang-1 levels increased but were coincident with a progressive decrease in Ang-2 levels. Ang-2 has been implicated as being a natural angiostatic factor whose binding affinity for the Tie-2 receptor is comparable to that of Ang-1[28]. However, the increased Ang-2 expression 8 hrs after MI and than decreased expression 12 and 24 hrs after MI levels specifically support the argument for its playing an important role in early angiogenesis. Its increased presence may actually be pro-angiogenic in nature by effecting dissolution of surrounding matrix thereby setting up a suitable environment in which endothelial cell migration and capillary sprouting can occur. Furthermore, the surprisingly coincident yet opposite temporal trends in Ang-1 and Ang-2 levels suggest that although they are antagonists at the receptor level, regulation of their protein levels in response to ischaemia runs much deeper and seems to indicate modulatory control at the transcriptional and/or translational level. Whether Ang-1 expression is suppressed by Ang-2 or they are both under the control of an as-of-yet unknown factor remains to be established.

Again, it is likely that sildenafil exerts a pro-angiogenic action through various angiogenic factors [15, 29, 30]. The most notable being VEGF, which has been chiefly associated with initiating the process of angiogenesis through the recruitment and proliferation of endothelial cells. The other angiogenic factor stimulation, such as Ang-1 by sildenafil [15] has been found to regulate the maturation of new blood vessels from the proliferating endothelial cells. Although the mechanism by which sildenafil induces myocardial VEGF expression remains unclear, several scenarios are possible. First, sildenafil activates K_{ATP} channels and induces nitrate-like effects. It might increase the production of interstitial adenosine through nitric oxide or K_{ATP} channel-mediated activation of well-known factor named ecto-5′nucleotidase [31]. Adenosine is thought to increase VEGF protein and mRNA expression by A2R. The endogenous adenosine can account for the basal VEGF secretion by myocardial vascular smooth muscle cells and probably be an important factor for the vasculature [32]. Secondly, sildenafil, which activates phosphorylation and activation of eNOS expression, might contribute to the up-regulation of VEGF expression. It has been reported that deficiency of eNOS resulted in a marked impairment of myocardial capillary development and reduction of VEGF expression in the neonatal mouse myocardium [33]. In addition, Zhang *et al.* has reported that nitric oxide enhances angiogenesis *via* the synthesis of VEGF and cGMP in a stroke model [34]. Finally, enhancement of Trx followed by HO-1 expression by sildenafil might contribute to the up-regulation of VEGF expression. Recent reports suggests a contribution of Trx system in the up-regulation of HO-1 protein levels as well as HO-1 [35, 36] promoter activity under conditions associated with inflammation and increased oxidative stress. The present study is in agreement with an earlier report demonstrating overexpression of HO-1 which augments the angiogenic effect of endothelial cells [37]. It is also

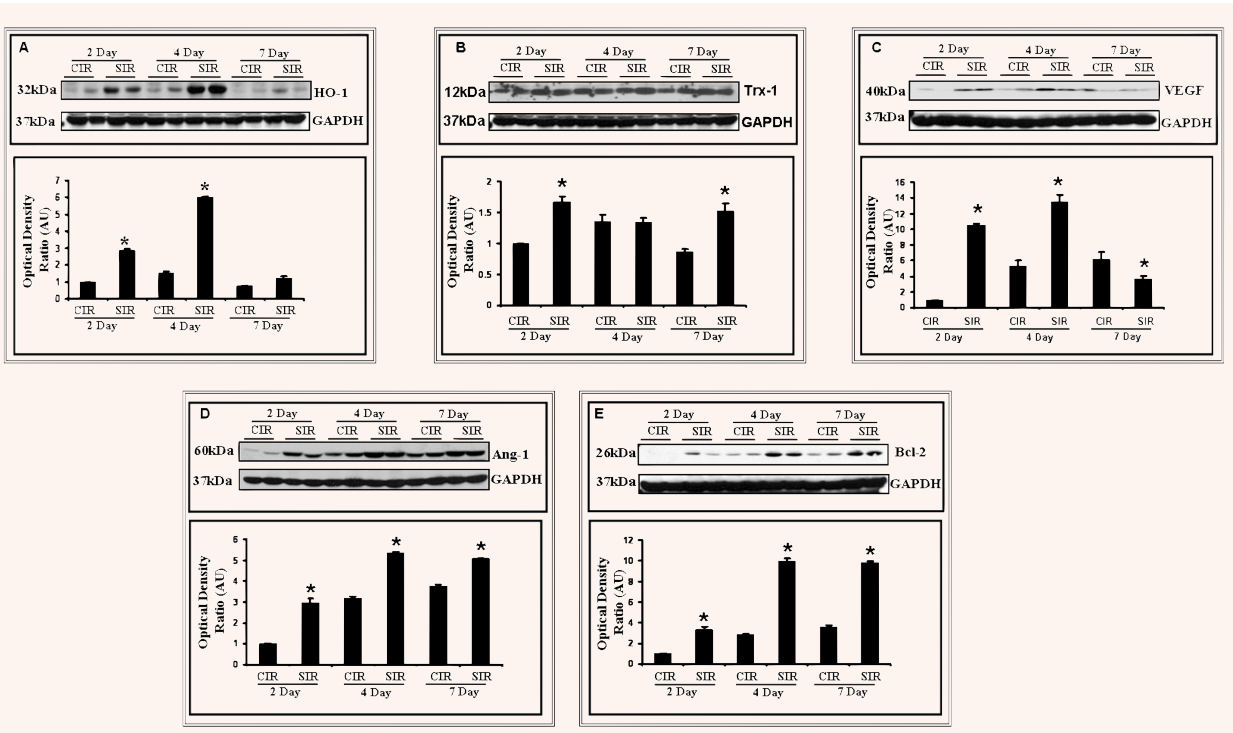

Fig. 6 Effect of sildenafil on the expression of HO-1, Trx-1, VEGF, Ang-1 and Bcl2. **A**, **B**, **C**, **D** and **E** show representative Western blots for the effects of sildenafil on the expression of (**A**) HO-1; (**B**) Trx-1; (**C**) VEGF; (**D**) Ang-1 and (**E**) Bcl-2 in CIR and SIR following 2, 4, 7 days of reperfusion. HO-1, Trx-1, VEGF, Ang-1 and Bcl-2 were expressed as 32, 12, 40, 60 and 26 kD respectively. GAPDH was used as the loading control. Bar graph below each Western blot shows the optical density ratio in arbitrary units. * $P < 0.05$ compared with CIR. CIR represents control IR group and SIR represents sildenafil IR group.

known that activation and overexpression of HO-1 leads to the up-regulation of VEGF synthesis. Moreover, role of HO-1 related improvement in cardioprotection against ischaemia–reperfusion injury and ventricular fibrillation has been reported [38–40]. Our findings suggest that the VEGF and Bcl-2 were up-regulated in response to sildenafil and functioned endogenously to promote myocardial angiogenesis. This contention is also supported by studies in which supplemental VEGF and Bcl-2 *in vitro* and *in vivo* have been shown to induce angiogenesis in various tissues and cell cultures [41]. Moreover, it is also demonstrated earlier that sildenafil stimulates angiogenesis through protein kinase G/MAPK pathway [42].

The cardioprotective effect of sildenafil [16] was significantly attenuated by SNPP (HO-1 enzyme inhibitor) which was related to decreased VEGF expression [15]. It has been reported that the redox protein Trx-1 increases hypoxia-inducible factor-1α (HIF-1α) protein expression under both normoxic and hypoxic conditions. This is found to be associated with augmented VEGF formation and increased tumour angiogenesis *in vivo* [43]. The hypoxia-inducible factor-1α complex influences the expression of many genes including VEGF [44]. Trx is an endogenous multi-functional protein with a redox-active disulfide/dithiol within the conserved active site sequence. It is also a scavenger of reactive oxygen species (ROS). Again, Trx-1 has not only antioxidant effect but also anti-apoptotic effect [45]. Therefore sildenafil-mediated induction of Trx-1 in ischaemic myocardium is promising.

Data in the present study also revealed the ability of sildenafil pre-treatment to exert a pronounced and marked effect in maintaining left ventricular contractile reserve. Differences in left ventricular contractile function between operated groups were not apparent from baseline functional criteria, as might be expected in the early post-MI state when intrinsic compensatory mechanism are activated. Sildenafil significantly enhanced level of contractile function. The effect on contractile reserve observed in the sildenafil group at 1 week post-op may be attributed at least partially to the concurrent increase in capillary density. While the increase is clearly suggestive of new capillary growth, the increased maintenance of capillary density at 2 and 4 day post-op in the sildenafil group is perhaps best explained as enhanced endothelial cell survival early after 30 min of LAD occlusion and 24 and 48 hrs of reperfusion. The marginal yet significant increase in arteriolar density in the sildenafil group compared to non-treated group of rats indicated

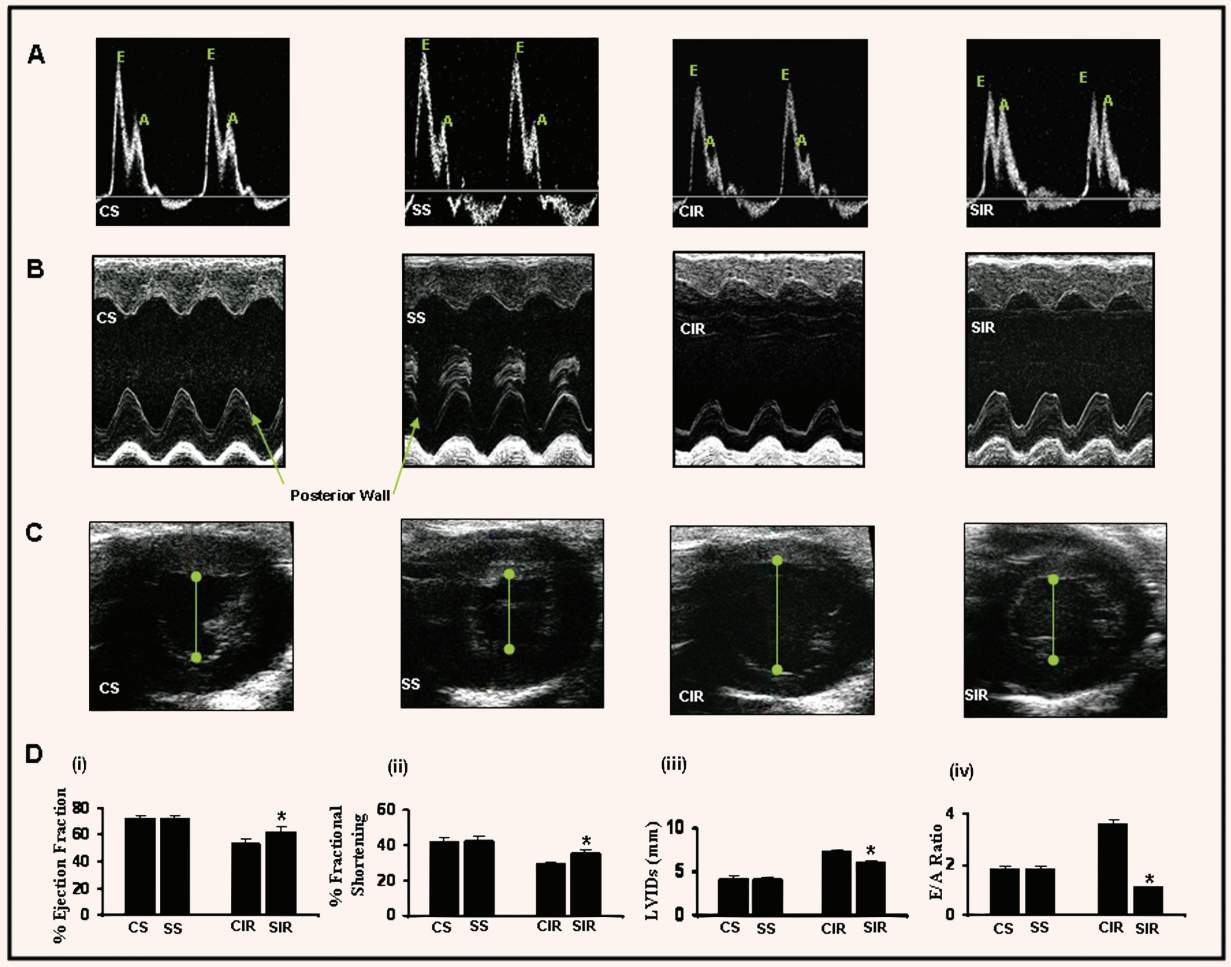

Fig. 7 Echocardiographic analysis. (**A**) Representative pulse wave Doppler of mitral wave velocity showing improved E/A in sildenafil IR group with minimal diastolic dysfunction compared to control IR having restrictive pattern of filling. Normal diastolic function was observed in CS and SS groups. (**B**) M-mode of echocardiograph pictures showing improved wall motion in SIR compared to CIR and was represented by increased fractional shortening and ejection fraction in sildenafil-treated group. (**C**) Parasternal short axis view showing dilated chamber in CIR compared to SIR. (**D**) Echocardiographic measurements in control and sildenafil-treated rats following 45 days of reperfusion. (*i*) Ejection fraction (%), (*ii*) fractional shortening (%), (*iii*) left ventricular inner diameter (LVIDs) (mm), (*iv*) E/A ratio. $*P < 0.05$ represents significant difference between CIR and SIR groups. Significant difference was not observed in CS and SS groups. LVIDs represents left ventricular internal diameter in systole. $n = 6$ in each group. CS, control sham; SS, sildenafil sham; CIR, control IR group and SIR, sildenafil IR group.

that the process of neovascularization is not restricted to the capillary level but extends to the arteriolar level as well. The blood flow data demonstrated increased blood supply to the sildenafil-treated myocardium after 14 days post-op compared to the non-treated group.

The other major aim of the present study was to probe the capacity of myocardium to respond to ischaemia in the presence of sildenafil. It has been well established in previous studies [46] that sildenafil attenuates the remodelling and reverses left ventricular hypertrophy when animal hearts were subjected to pressure overload. Moreover, it has also been shown in a recent human study [47] that sildenafil directly influences cardiac function in healthy human beings by suppressing β-adrenergic-stimulated systolic function while having minimal effect under resting conditions. In our previous study we documented that at 0.05 mg/kg (given 60 min. prior ischaemia) and to some extent at 0.01 mg/kg sildenafil provided significant cardioprotection as evidenced by improved ventricular recovery, a reduced incidence of ventricular fibrillation and decreased

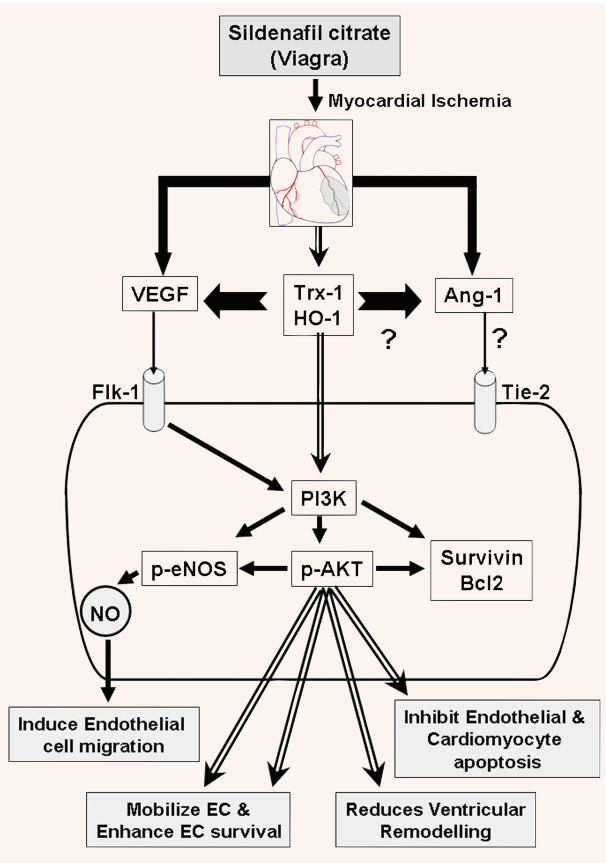

Fig. 8 Schematic diagram. The mechanism of sildenafil-induced neovascularization in the infarcted myocardium.

myocardial infarction in a isolated working rat heart model [1]. Also Bremer *et al.* [48] have also shown the effect of sildenafil on the acute haemodynamic effect and the left ventricular function.

In the present study, sildenafil-treated animals showed significant increase in EF and FS compared to control animals. It was also noted that E/A ratios were much better and statistically significant in sildenafil-treated animals when compared to controls, indicating sildenafil minimizes the left ventricular diastolic dysfunction in post infarct period (Fig. 6A–D). Also, left ventricular systolic function (EF & FS) and left ventricular chamber size were preserved in the sildenafil-treated group compared to non-treated. Thus, these results show the enormous potential of sildenafil to reorganize ischaemic vasculature. Sildenafil therapy could potentially be used in combination with conventional revascularization procedures to reduce infarct size and cell death and enhance capillary perfusion. The proposed mechanism of sildenafil-induced neovascularization and reduced ventricular remodelling is shown in Fig. 8.

In conclusion, sildenafil is a very promising drug for the induction of clinically relevant therapeutic regeneration of infracted myocardium. This may involve several growth factors including VEGF, Ang-1, Trx and HO-1 capable of generating new vessels in an ischaemic milieu which increases myocardial revascularization and potentially improves ventricular function by restoring blood flow to the viable myocardium.

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