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Therapeutic Evaluation of Recombinant Human Erythropoietin (rhEPO) In Isoproterenol Induced Myocardial Infarction in Wistar Rats

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Abstract: Recombinant human erythropoietin (rhEPO) is an effective treatment for myocardial infarction (MI) by promoting cardiac repair. However, additional investigations are required to determine its therapeutic value. The present study aims to investigate the effect of rhEPO in isoproterenol- (ISO-) induced myocardial infarction in Wistar rats. The efficacy of EPO pre- and post-therapy, as evaluated by histology, histomorphometry, and immunohistochemistry analysis, forms the novelty of the present study. Adult male Wistar Albino rats were taken in four groups (Group1- control, Group2 - ISO induced MI, Group 3-ten days rhEPO pre-treatment + ISO induction, and Group4-ISO induction + single dose rhEPO post-treatment), respectively. While Group 1 rats served as normal controls, Group 2 rats received a single ISO-isoproterenol (75 mg/kg b.w) on the final day of the trial. The rats in Group 3 received rhEPO (5000 IU/kg i.p) injections once daily for 10 days and a single dosage of isoproterenol (75 mg/kg i.p) on the 10th day. The group 4 rats received a single injection of ISO (75 mg/kg i.p.) followed by a single injection of rhEPO (5000 IU/kg i.p.) at a 2-hour interval. The rats were sacrificed, and samples were taken for further study. The therapeutic potential of rhEPO, i.e., ten days' pre-treatment before ISO induction, as well as the efficacy of a single dose of rhEPO post-treatment following ISO-induced myocardial damage, was investigated by immunohistochemical alterations of collagen, caspase1, p-SMAD3, and ASC proteins in the heart tissue. The histo pathological changes were evaluated in the heart tissues of study group rats. The immuno histo chemistry modifications reveal that erythropoietin post-therapy is more effective than pre-treatment against MI. However, histological abnormalities in cardiac tissues confirm it. As a result, rhEPO is more effective against MI after treatment than before treatment.

Key Words: Isoproterenol; Myocardial infarction; Erythropoietin; Wistar rats; ASC Protein.

1. INTRODUCTION

Myocardial infarction (MI) is one of the major cardiovascular disorders in which one or more coronary arteries that provide oxygen-rich blood to the heart muscle abruptly get clogged.[1] The most prevalent cause of myocardial infarction is partial or full epicardial coronary artery blockage from plaques prone to rupture or erosion, accounting for around 70% of fatal occurrences.[2] A prolonged blockage of blood supply (particularly the coronary artery) to myocardial tissue (i.e., hypoxia) results in myocyte death, which eventually leads to MI. However, the pathophysiology behind MI remains unknown. Despite this, few studies on MI have shown that hypoxia, necrosis, mitochondrial dysfunction, apoptosis, oxidative stress, and inflammation are key contributors.[3] According to Liu et al., Myocardial infarction symptoms include chest pain that extends from the left arm to the neck, breathing difficulty, sweat, nausea, vomiting, inconsistent heartbeat, anxiety, exhaustion, weakness, stress, sadness, and other variables.[4] Some people with diabetes and older people may get a silent MI. It may also manifest with several uncommon symptoms, such as stomach ache. Importantly, men and women experience different symptoms.In most cases, MI may present silently, which makes it difficult to treat. Myocardial infarction is still one of the major causes of mortality worldwide, and some people are still unaware of its risk factors due to a lack of information.Isoproterenol is often used to cause experimental MI in rats. Isoproterenol 4-[1-hydroxy-2-(isopropylamine) ethyl] benzene-1,2-diol hydrochloride (ISO) is a synthetic catecholamine and nonselective --adrenergic agonist that has been shown to cause significant stress in the myocardium and MI when administered at supramaximal doses.[5] This effect is accomplished by causing myocardial oxidative stress, inflammation, and calcium overload via activation of 1-adrenergic receptors in the heart.[6] Isoproterenol causes myocardial necrosis in the rat model, which leads to cardiac dysfunction, increased lipid peroxidation, and myocardial lipid levels, as well as altered cardiac enzyme and antioxidant activity. Pathophysiological and morphological changes in the heart of the myocardial necrotic rat model are the same as in human MI.[7] Exploring pharmaceutical strategies to treat ISO-induced cardiac anomalies could be beneficial in avoiding the onset and progression of MI.[8]Rajadurai*et al*., usedIsoproterenol (ISO) induced myocardial infarction (MI) in male Wistar rats to investigate the prevention of MI using naringin.[9]Erythropoietin (EPO) is a 165 amino acid glycoprotein hormone generated by the fetal liver and adult kidney that belongs to the broad and diverse cytokine superfamily.[10] It is primarily used in the treatment of myocardial infarction.[11] The first experiment was published on animal experiments in the model of persistent coronary artery occlusion.[12]Currently, recombinant DNA technology is used to manufacture EPO in Chinese hamster ovary cells (Recombinant Erythropoietin rhEPO). The human erythropoietin gene was isolated and then inserted into and produced by grown mammalian cells capable of creating an infinite amount of the hormone.[13] The therapeutic potential of rhEPO in cardioprotection was revealed in models of myocardial ischemia where EPO prevented apoptosis and augmented survival of cardiac myocytes.[14] Numerous research studies have revealed promising outcomes from using rhEPO to treat myocardial infarction. However, further studies are needed to explore the effectiveness of rhEPO in treating MI. This study aims to examine the efficacy of rhEPO against MI. The present study is focused on the pre and post-treatment of rhEPO in isoproterenol-induced MI in male Wistar by determining Immunohistochemical alterations and histopathological changes.

2. MATERIALS AND METHODS

2.1. Animals

Male Wistar rats (250-300gm) procured from Biogen Laboratory Animal Facility, Bengaluru, were maintained in polypropylene cages at 23 ± 20°C with a relative humidity of 40–60%, and the natural light-dark cycle was maintained throughout the research. The animals were fed with a commercial pellet diet (Krishna Valley Agrotech, LLP, Sangli, Maharashtra) and filtered water *ad libitum*. This study was conducted per the guidelines of the Committee for the Control and Supervision of Experiments on Animals (CCSEA, India). It was approved by the Institutional Animal Ethics Committee (SU/CLAR/RD/003/2022) of Saveetha Medical College.

2.2. Drugs and Chemicals

The rhEPO drugs were procured from Intas Pharmaceuticals Ltd., Ahmedabad, India. Each prefilled syringe of 1.0 mL contained 4000 IU of recombinant human Erythropoietin injection IP (RENOCEL 4000). The isopropanol (Isolin – 2mg/mL vial) was procured from Samarth Life Sciences Pvt Ltd., Mumbai, India. All other reagents and chemicals used in the study were of analytical grade.

2.3. Experimental Animals

The experimental animals were divided into four groups; each group had six animals. While group 1 rats served as normal control, the group 2 rats were administered a single intraperitoneal (i.p) injection of ISO-isoproterenol (75 mg/kg b.w) on the last day of experimentation (i.e., day of sacrificing animals). The group 3 rats (therapeutic drug pre-treated) received rhEPO (5000 IU/kg i.p) injections continuously once daily for 10 days. After receiving the last dose of rhEPO injection on the 10th day, a single dose of isoproterenol (75 mg/kg i.p) was injected the next day and then sacrificed after a 2-hour duration. On the day of sacrificing all rats (11th day), the group 4 rats received a single injection of ISO (75 mg/kg i.p) first followed by a single injection of rhEPO (5000 IU/kg i.p) as post-treatment at 2 hrs time interval. One hour after rhEPO injections, the group 4 rats were also sacrificed.

2.4. Induction of Experimental MI

The myocardial infarction (MI) was induced in male Wistar rats based on the previously published work.[14]Briefly, the MI was induced by injecting a single dose of isoproterenol (75 mg/kg) into rats through an intraperitoneal route of administration. While MI is induced in rodents either by subcutaneous or intraperitoneal mode of ISO administration, the present study adopted the intraperitoneal mode of injection.

2.5. Experimental Design

The rats were divided into the following study groups:
Group I: Normal control (No treatment);
Group II: Myocardial infarction induction with isoproterenol (ISO) (75 mg/kg i.p)[15]
Group III: rhEPO (5000 IU/kg i.p) pre-treatment for 10 days + ISO (75mg/kg i.p)[15]
Group IV: ISO (75mg/kg i.p) + rhEPO post-treatment (5000 IU/kg i.p)[15]

At the end of the experimental protocol, the animals were anesthetized using isoflurane (Raman and Weil, Mumbai), and blood samples were withdrawn following retro-orbital puncture using hematocrit capillary tubes. The blood was collected into vacutainers (Clot activator). Subsequently, the serum was collected by allowing the blood to clot for 15-30 min at RT followed by centrifugation at 3,500 rpm for 10 min in a cooling centrifuge (REMI CPR-24PLUS, India) and preserved for biochemical investigations (which are not included in this article). The animals were trans-cardially perfused with normal saline followed by a neutral buffered formalin solution. The heart tissues were carefully removed and processed for histo-pathological and immunohistochemical examination.

2.6. Histopathological Examination and histomorphometric analysis of heart tissues

Following animal sacrifice, the heart tissue was quickly dissected out and washed with saline before being fixed in a 10% neutral buffered formalin solution. The fixed tissues were immersed in paraffin and sliced into 5 μm thick serial sections using a rotary microtome and later stained with hematoxylin and eosin (H&E) dye for histopathological examination under a light microscope (Olympus BX-51). Photomicrographs were obtained with a digital camera mounted to the microscope. The pathologist who performed the histological examination of stained slides was not aware of the animal groups.The histomorphometric analysis was performed on an H&E stained slide at 40× magnification. From each slide, ten microscopic fields were randomly selected. For each microscopic field, a grid with regular spaced points was used to count the number of cardiomyocytes. The sections were scored using a semiquantitative scale to evaluate the degree of cellular degeneration, inflammatory cell infiltration, interstitial edema, and necrosis. Histopathological damage was scored according to the previous reports as 0: no damage; 1: damage in <10% of all cardiomyocytes; 2: damage in 10%-30% of all cardiomyocytes; damage in >30% of all cardiomyocytes. The results are depicted as graphs (Figure 1E) and tables (2 and 3).

2.7. *Immunohistochemical studies of collagen 1, caspase 1, p-SMAD3, and ASC proteins*

2.7.1. *Collagen 1 Immunostaining*

Immunolocalization of proteins (antigen) in rat heart tissue was done using the `indirect peroxidase' method. Immunohistochemical staining on Collagen type 1 was performed. For this purpose, an HRP/DAB detection IHC kit was used according to the manufacturer's protocol. Paraffin was removed in xylene, and the sections were dehydrated through an alcohol series. After two rinses in PBS for 5 min each, the endogenous peroxidase activity was removed by incubation in 3% hydrogen peroxide for 30 min at room temperature. The non-specific binding sites were blocked by incubation with normal goat serum (3 drops in 3% BSA in PBS) for 30 min. After antigen retrieval (100 × Citrate Buffer) for 20 min in a domestic pressure cooker and blocking non-specific binding sites with protein block, the sections were immunoreactive with 20 μg/mL primary antibodies against Collagen 1 (Sigma Aldrich Company) overnight at 4°C, respectively. Following this, the incubation was done for 60 min at room temperature using a primary antibody (1:100). For 60 min at room temperature, sections were incubated with biotinylated anti serum (goat antiserum to rabbit IgG 1:100 dilution) after rinsing with PBS. Then, the sections were incubated in the working streptavidin HRP solution for 60 minutes at room temperature and washed in three changes of PBS. Finally, the sections were incubated with DAB-hydrogen peroxide for 30 minutes, washed in water, counterstained, and viewed under a light microscope (Olympus BX-51). The quantification of the intensity was measured using the image analysis software tool.

2.7.2. *Caspase-1 Immunostaining*

Immunolocalization of proteins (antigen) in rat heart tissue was done using the `indirect peroxidase' method. Immunohistochemical staining on caspase-1 was performed. For this purpose, an HRP/DAB detection IHC kit was used according to the manufacturer's protocol. Paraffin was removed in xylene, and the sections were dehydrated through an alcohol series. After two rinses in PBS for 5 min each, the endogenous peroxidase activity was removed by incubation in 3% hydrogen peroxide for 30 min at room temperature. The non-specific binding sites were blocked by incubation with normal goat serum (3 drops in 3% BSA in PBS) for 30 min. After antigen retrieval (100 × Citrate Buffer) for 20 min in a domestic pressure cooker and blocking non-specific binding sites with protein block, the sections were immunoreactive with 10 μg/mL primary antibodies against caspase-1 (Sigma Aldrich Company) overnight at 4°C, respectively. Following this, the incubation was done for 60 min at room temperature using a primary antibody (1:100). For 60 min at room temperature, sections were incubated with biotinylated anti serum (goat antiserum to rabbit IgG 1:50 dilution)after rinsing with PBS. Then, the sections were incubated in the working streptavidin HRP solution for 60 minutes at room temperature and washed in three changes of PBS. Finally, the sections were incubated with DAB-hydrogen peroxide for 30 minutes, washed in water, counterstained, and viewed under a light microscope (Olympus BX-51). The quantification of the intensity was measured using the image analysis software tool.

2.7.3. *Smad3 Immunostaining*

Immunolocalization of proteins (antigen) in rat heart tissue was done using the `indirect peroxidase' method. Immunohistochemical staining on smad3 was performed. For this purpose, an HRP/DAB detection IHC kit was used according to the manufacturer's protocol. Paraffin was removed in xylene, and the sections were dehydrated through an alcohol series. After two rinses in PBS for 5 min each, the endogenous peroxidase activity was removed by incubation in 3% hydrogen peroxide for 30 min at room temperature. The non-specific binding sites were blocked by incubation with normal goat serum (3 drops in 3% BSA in PBS) for 30 min. After antigen retrieval (100 × Citrate Buffer) for 20 min in a domestic pressure cooker and blocking non-specific binding sites with protein block, the sections were immunoreactive with 10 μg/mL primary antibodies against smad3 (Sigma Aldrich Company) overnight at 4°C, respectively. Following this, the incubation was done for 60 min at room temperature using a primary antibody (1:100). For 60 min at room temperature, sections were incubated with biotinylated anti serum (goat antiserum to rabbit IgG 1:50 dilution) after rinsing with PBS. Then, the sections were incubated in the working streptavidin HRP solution for 60 minutes at room temperature and washed in three changes of PBS. Finally, the sections were incubated with DAB-hydrogen peroxide for 30 minutes, washed in water, counterstained, and viewed under a light microscope (Olympus BX-51). The quantification of the intensity was measured using the image analysis software tool.

2.7.4. *ASC Immunostaining*

Immunolocalization of proteins (antigen) in rat heart tissue was done using the `indirect peroxidase' method. Immunohistochemical staining on ASC was performed. For this purpose, an HRP/DAB detection IHC kit was used according to the manufacturer's protocol. Paraffin was removed in xylene, and the sections were dehydrated through an alcohol series. After two rinses in PBS for 5 min each, the endogenous peroxidase activity was removed by incubation in 3% hydrogen peroxide for 30 min at room temperature. The non-specific binding sites were blocked by incubation with normal goat serum (3 drops in 3% BSA in PBS) for 30 min. After antigen retrieval (100×Citrate Buffer) for 20 min in a domestic pressure cooker and blocking non-specific binding sites with protein block, the sections were immunoreactive with 10 µg/mL primary antibodies against ASC (Sigma Aldrich Company) overnight at 4°C, respectively. Following this, the incubation was done for 60 min at room temperature using a primary antibody (1:1000). For 60 min at room temperature; sections were incubated with biotinylated anti serum (goat antiserum to rabbit IgG 1:00 dilution) after rinsing with PBS. Then, the sections were incubated in the working streptavidin HRPsolution for 60 minutes at room temperature and washed in three changes of PBS. Finally, the sections were incubated with DAB-hydrogen peroxide for 30 minutes, washed in water, counterstained, and viewed under a light microscope (Olympus BX-51). The quantification of the intensity was measured using the image analysis software tool.

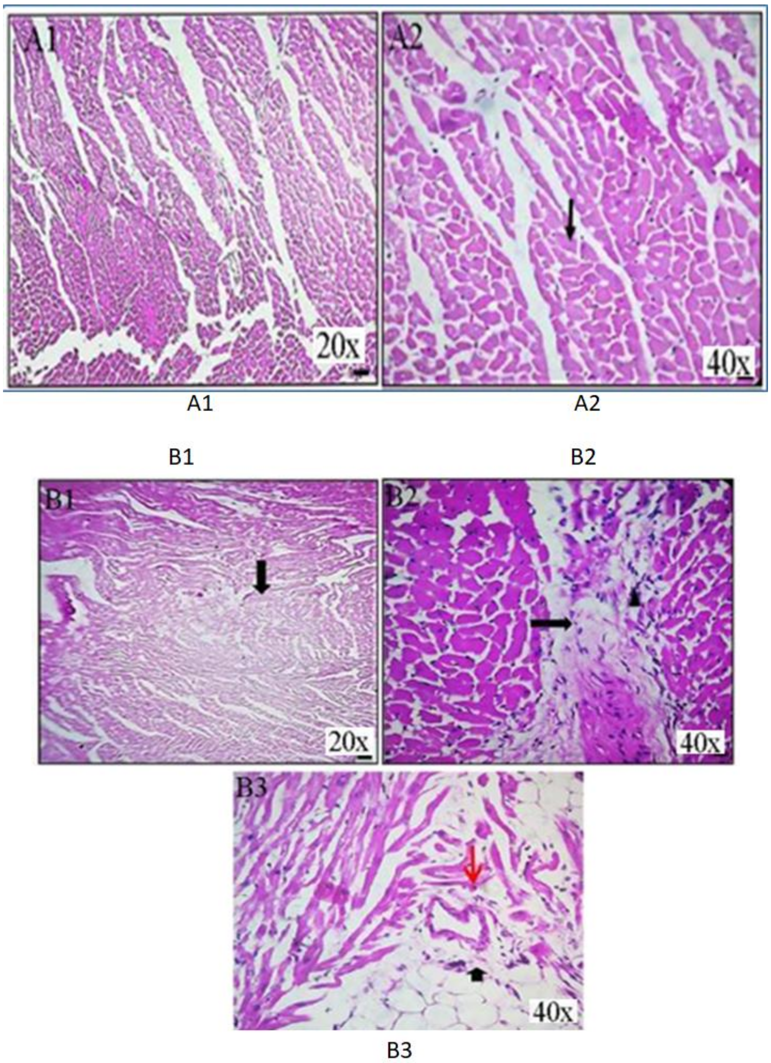
2.8. Statistical analysis

The data were expressed as mean, standard deviation, and standard error. The mean values were compared by one-way analysis of variance, and once they were found statistically significant, multiple comparison tests were done using the Bonferroni 't' test. A probability of 0.05 or less was considered statistically significant. SigmaPlot 14.5 version (Systat Software Inc., San Jose, USA) was used for statistical analysis and graph plotting.

3. RESULTS

3.1. Histopathological changes in the heart tissue with histomorphometry report

The histological changes visualized in the heart tissues of the various groups are depicted in Figure 1 at two different magnifications (20x and 40x respectively).



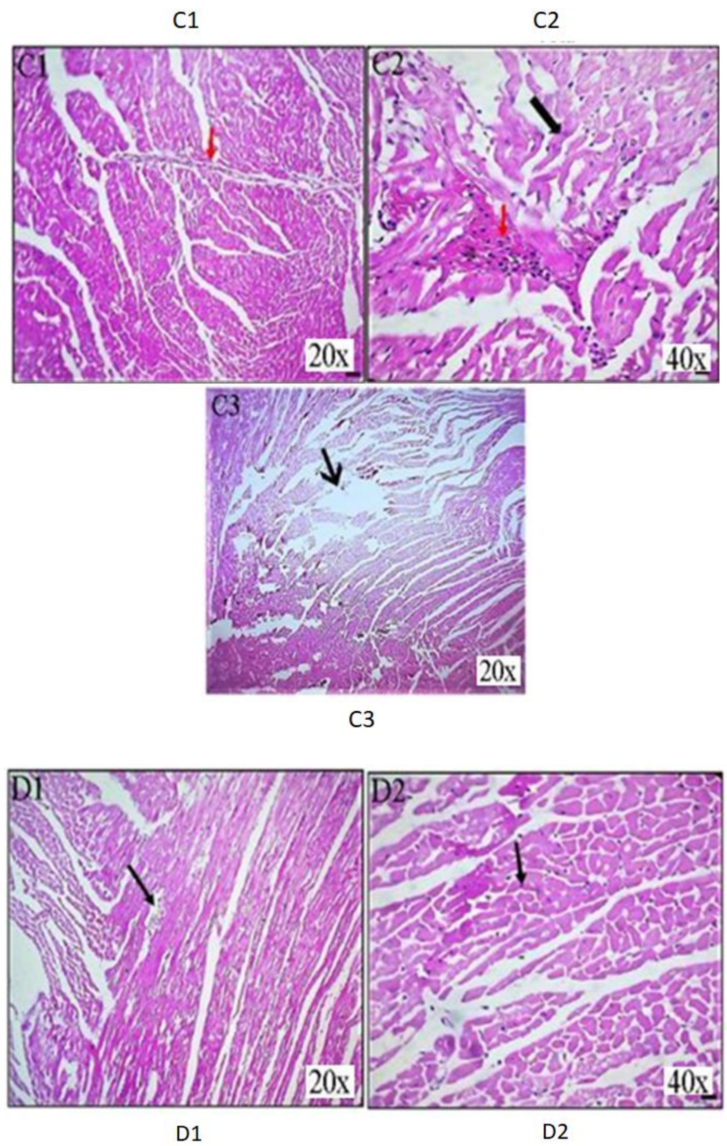


Fig 1: Histopathological changes in the heart tissues of the various groups: A-control group, B-MI induced group, C- rhEPOpretreatment group before MI and D - rhEPO post-treatment following MI.

The images (Fig 1) of normal control group (Panel A) depicted normal architecture of cardiac tissues, normal myocytes with single nucleus. The images of MI group (Panel B) showed myofibrillar loss (broad arrow mark), necrosis (arrow head) inflammatory cell infiltration (red arrow) and very less interstitial collagen fibres. The images of thegroup that received rhEPOpretheray before MI induction (Panel C) showed less myofibrillar loss (broad black arrow), disruption of myocardial fibres (broad arrow mark) and inflammatory cell infiltration (small red arrow) seen. The histology photos of the rhEPOposttreated and MI induced group (Panel D) showed minimal inflammatory infiltrates (small arrow) and myocardial cell.The histology photos of the normal control group (Figure A1 & A2) depicted normal architecture of cardiac tissues, normal myocytes with single nucleus (small arrow). The histology photos of the MI induced group (B1, B2 & B3) showed myofibrillar loss (broad arrow mark), necrosis (arrow head) , inflammatory cell infiltration (red arrow) and very less interstitial collagen fibres. The images of the rhEPOpretreatment (10 days) group before MI (Figure C1 & C2) showed less myofibrillar loss (broad black arrow), disruption of myocardial fibres (broad arrow mark) and inflammatory cell infiltration (small red arrow) whereas the images of the rhEPO post-treatment following MI revealed minimal inflammatory infiltrates (small arrow) and myocardial cell (Figure D1 & D2) and significant reduction in cardiac damage. The quantification of cardiomyocyte number is shown as bar graph (Figure 1E)

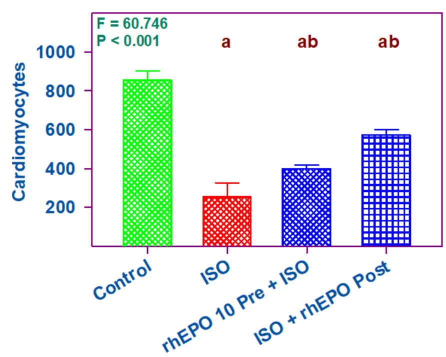


Fig 1E: Comparative effect of erythropoietin (rhEPO) pre-treatment and post-treatment on isoproterenol (ISO) induced changes on cardiomyocytes in Wistar rats.

Values are mean ± SE (n = 3 each), Pre = 10 days pre-treatment with erythropoietin followed by isoproterenol on the next day.Post = Isoproterenol followed by erythropoietin treatment after 1 hr.The 'F' and 'P' values are by one way ANOVA with Bonferroni 't' test for multiple comparison.[a]Significantly different from control.[b]Significantly different from isoproterenol.[c]Significantly different from erythropoietin pre-treatment + isoproterenol.

The histomorphometric scoring (grade) of inflammatory cell infiltration, interstitial edema, cellular degeneration and necrosis are depicted graphically (Figure 1F) and in table 2 and 3 respectively.

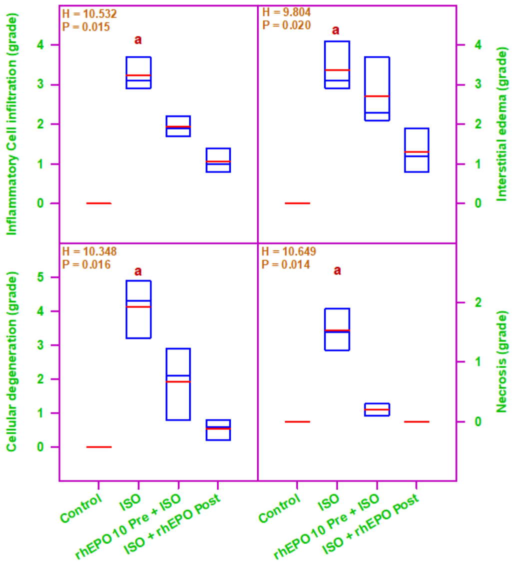


Fig 1F: Comparative effect of erythropoietin (rhEPO) pre-treatment and post-treatment on isoproterenol (ISO) induced histomorphometric changes in Wistar rats.

Values are mean ± SE (n = 3 each); Pre = 10 days' pre-treatment with erythropoietin followed by isoproterenol on the next day.Post = Isoproterenol followed by erythropoietin treatment after 1 hr.The 'F' and 'P' values are by one-way ANOVA with Bonferroni 't' test for multiple comparison.[a]Significantly different from control.

The mean, standard deviation and standard error of collagen 1, caspase 1, p-Smad3 and positive cells (%) are given in Table 1.

Table 1: Comparative effect of erythropoietin (rhEPO) pre-treatment and post-treatment on isoproterenol (ISO) induced changes in Wistar rats.

S.No.	Parameter	Groups	Mean	SD	SE	Statistics
1	Collagen 1 positive cells %	Control	18.83	6.63	3.83	F = 28.641 P < 0.001
		ISO	66.53	15.08	8.70	
		rhEPO 10 Pre + ISO	50.97	1.99	1.15	
		ISO + rhEPO Post	12.10	2.55	1.47	
2	Caspase 1 positive cells %	Control	23.5	2.10	1.21	F = 74.829 P < 0.001
		ISO	70.77	2.63	1.52	
		rhEPO 10 Pre + ISO	48.70	4.52	2.61	
		ISO + rhEPO Post	22.57	7.30	4.21	
3	P-Smad3 positive cells %	Control	5.77	2.66	1.54	F = 95.787 P < 0.001
		ISO	79.00	3.26	1.88	
		rhEPO 10 Pre + ISO	59.70	9.69	5.60	
		ISO + rhEPO Post	22.40	5.43	3.13	
4	ASC positive cells %	Control	0.803	1.296	0.748	F = 17.814 P < 0.001
		ISO	72.633	3.917	2.262	
		rhEPO 10 Pre + ISO	44.567	12.962	7.484	
		ISO + rhEPO Post	21.967	21.313	12.305	

n = 3 each; Pre = 10 days pre-treatment with erythropoietin followed by isoproterenol on the next day.Post = Isoproterenol followed by erythropoietin treatment after 2 hr.The 'F' and 'P' values are by one way ANOVA with Bonferroni 't' test for multiple comparison.The significance from control and ISO groups are given in Figure 1, 2 and 3.

Table 2: Comparative effect of erythropoietin (rhEPO) pre-treatment and post-treatment on

isoproterenol (ISO) induced changes on cardiomyocytes in Wistar rats.					
S.No.	Parameter	Groups	Mean	SE	Statistics
1	Cardiomyocytes (number)	Control	854	50	Given in Figure 1E
		ISO	186	37	
		rhEPO 10 Pre + ISO	418	23	
		ISO + rhEPO Post	572	28	

n = 3 each; Pre = 10 days pre-treatment with erythropoietin followed by isoproterenol on the next day.Post = Isoproterenol followed by erythropoietin treatment after 2 hr.The data was analysed by one-way ANOVA with Bonferroni 't' test for multiple comparison.

Table 3: Comparative effect of erythropoietin (rhEPO) pre-treatment and post-treatment on isoproterenol (ISO) induced histomorphological changes on cardiomyocytes in Wistar rats.

S.No.	Parameter	Groups	Median	Percentile	Statistics
1	Inflammatory Cell infiltration (grade)	Control	0	0 – 0	H = 10.532 P = 0.015
		ISO	3.1[a]	2.9 – 3.7	
		rhEPO 10 Pre + ISO	1.9	1.7 – 2.2	
		ISO + rhEPO Post	1	0.8 – 1.4	
2	Interstitial edema (grade)	Control	0	0 – 0	H = 9.804 P = 0.020
		ISO	3.1 [a]	2.9 – 4.1	
		rhEPO 10 Pre + ISO	2.3	2.1 – 3.7	
		ISO + rhEPO Post	1.2	0.8 – 1.9	
3	Cellular degeneration (grade)	Control	0	0 – 0	H = 10.348 P = 0.016
		ISO	4.3 [a]	3.2 – 4.9	
		rhEPO 10 Pre + ISO	2.1	0.8 – 2.9	
		ISO + rhEPO Post	0.6	0.2 – 0.8	
4	Necrosis (grade)	Control	0	0 – 0	H = 10.649 P = 0.014
		ISO	1.5	1.2 – 1.9	
		rhEPO 10 Pre + ISO	0.2	0.1 = 0.3	
		ISO + rhEPO Post	0	0 – 0	

n = 3 each, Pre = 10 days pre-treatment with erythropoietin followed by isoproterenol on the next day.Post = Isoproterenol followed by erythropoietin treatment after 2 hr.The data was analysed by Kruskal Wallis one-way ANOVA on ranks with Tukey's multiple comparison test.[a]Significantly different from control.[b]Significantly different from isoproterenol; [c]Significantly different from erythropoietin pre-treatment + isoproterenol.

The results of cardiomyocytequantificatin (count) revealed significant decrease ($p < 0.05$) in the ISO induced rats compared to control group. However, in EPO treatment groups (both pre and post therapy) the numbers of cardiomyocytes were considerably improved ($p < 0.05$). The pathological grade assessed by histomorphometry analysis showed signifincant changes in the ISO induced group compared with control ($p < 0.05$). Whereas in EPO treatment groups (both pre and post therapy) such changes were found to be reversed but not statistically significant.

3.2. Immunohistochemical alterations of collagen 1, caspase1, p-SMAD3, and ASC protein and in the heart tissue

The IHC results of collagen 1, caspase1, p-SMAD3, and ASC protein expression are depicted in Figures 2 to 5.

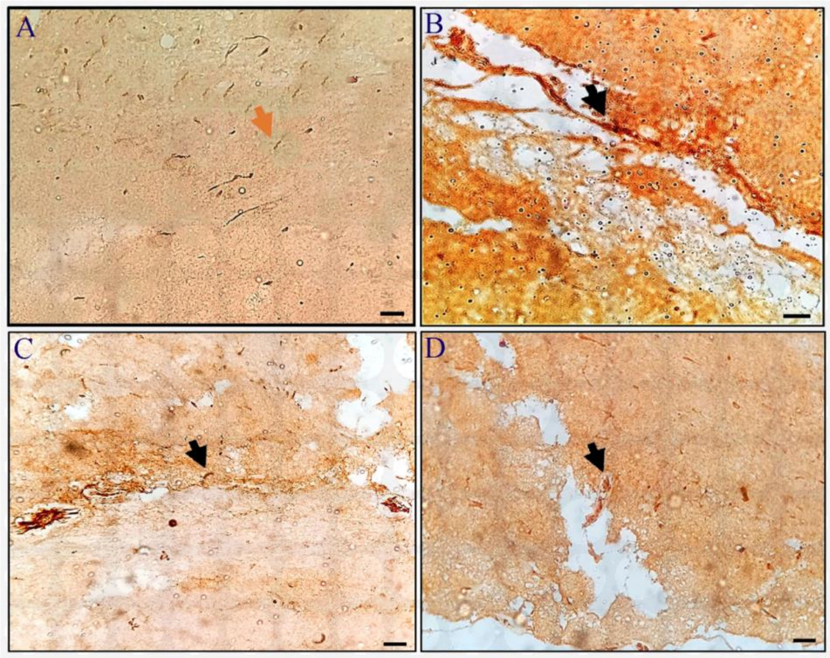


Fig 2: Immunohistochemically evaluation of collagen 1 expression levels in the rat heart tissues.

Panel A - Control group, Panel B – ISO, Panel C - rhEPO10 Pre + ISO,Pancel D - ISO + rhEPO post

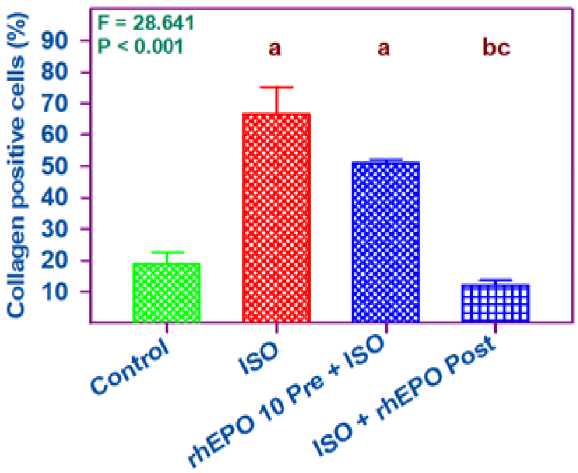


Fig 2E: Comparative effect of erythropoietin (rhEPO) pre-treatment and post-treatment on isoproterenol (ISO) induced changes in Wistar rats.

Values are mean ± SE (n = 3 each),Pre = 10 days pre-treatment with erythropoietin followed by isoproterenol on the next day,Post = Isoproterenol followed by erythropoietin treatment after 1 hr, Th 'F' and 'P' values are by one way ANOVA with Bonferroni 't' test for multiple comparison.[a]Significantly different from control,[b]Significantly different from isoproterenol,[c]Significantly different from erythropoietin pre-treatment + isoproterenol.

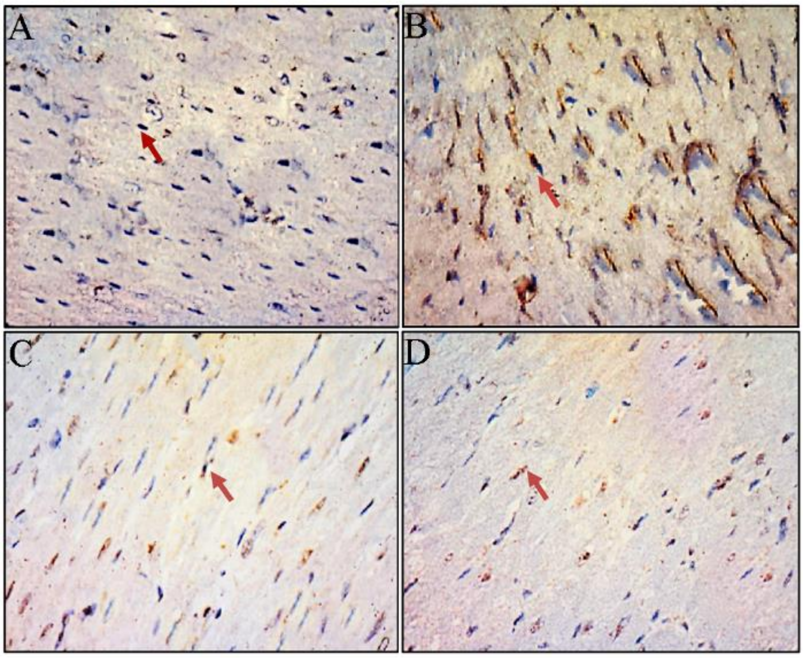


Fig 3: Immunohistochemical evaluation of caspase1 protein expression levels in the sections of rat heart tissues.

Panel A - Control group; Panel B – ISO; Panel C - rhEPO10 Pre + ISO; Panel D - ISO + rhEPO post

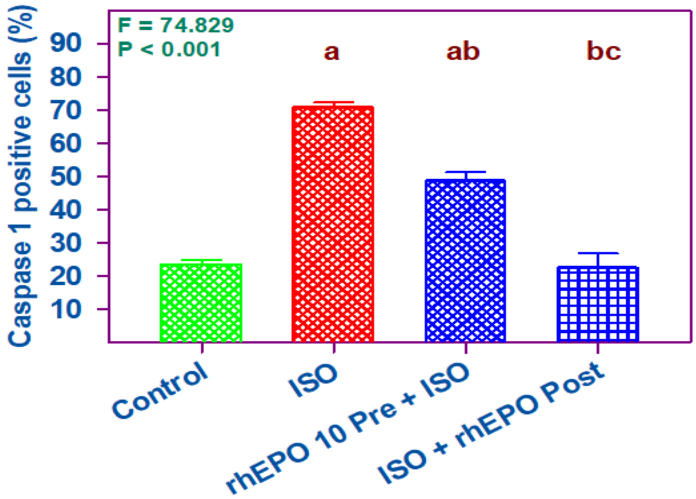


Figure 3E: Capase 1 expression on pre-treatment and post-treatment of erythropoietin (rhEPO) in isoproterenol (ISO) induced changes in Wistar rats.

Values are mean $\pm$ SE (n = 3 each); Pre = 10 days' pre-treatment with erythropoietin followed by isoproterenol on the next day.Post = Isoproterenol followed by erythropoietin treatment after 2 hrs.The 'F' and 'P' values are by one-way ANOVA with Bonferroni 't' test for multiple comparison.[a]Significantly different from control.[b]Significantly different from isoproterenol.[c]Significantly different from erythropoietin pre-treatment + isoproterenol.

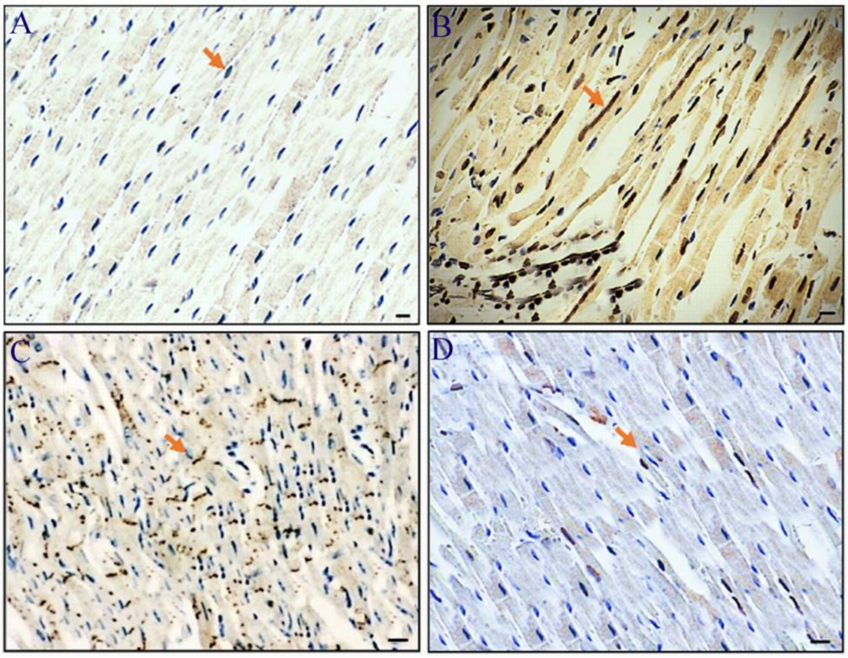


Fig 4: Immunohistochemical evaluation of p-SMAD3 protein expression levels in the sections of rat heart tissues.

Panel A - Control group; Panel B – ISO; Panel C - rhEPO10 Pre + ISO; Pancel D - ISO + rhEPO post

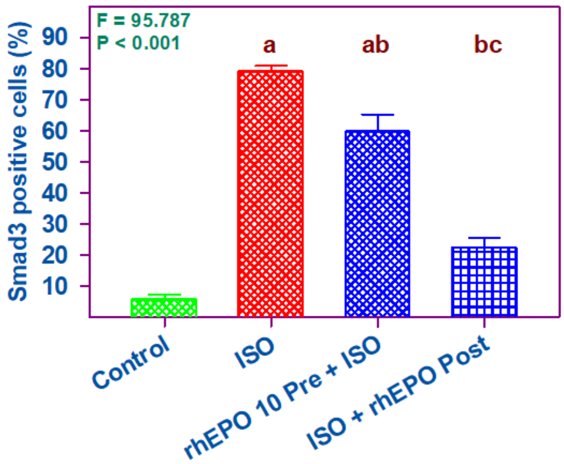


Fig 4E: Sdmad3 expression on pre-treatment and post-treatment of erythropoietin (rhEPO) in isoproterenol (ISO) induced changes in Wistar rats.

Values are mean + SE (n = 3 each); Pre = 10 days pre-treatment with erythropoietin followed by isoproterenol on the next day.Post = Isoproterenol followed by erythropoietin treatment after 2 hrs.The 'F' and 'P' values are by one-way ANOVA with Bonferroni 't' test for multiple comparison.[a]Significantly different from control.[b]Significantly different from isoproterenol.[c]Significantly different from erythropoietin pre-treatment + isoproterenol.

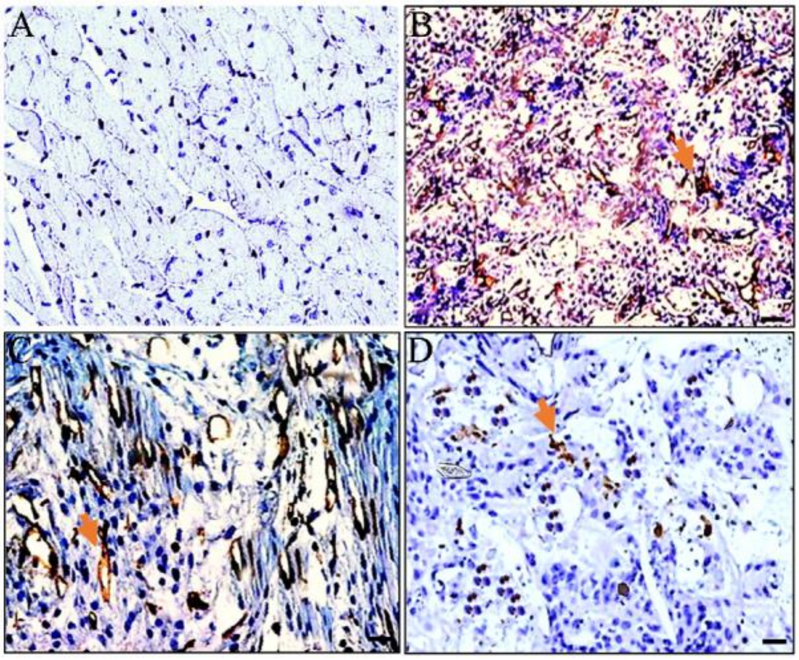


Fig 5: Immunohistochemical evaluation of ASC protein expression in the sections of rat heart tissue.

Panel A - Control group; Panel B – ISO; Panel C - rhEPO10 Pre + ISO; Pancel D - ISO + rhEPO post

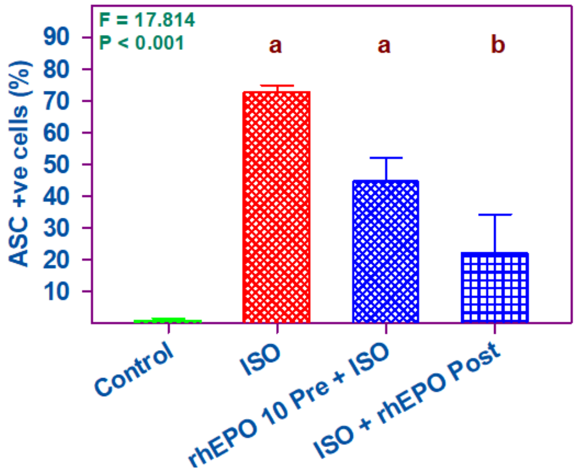


Fig 5E: Comparative effect of erythropoietin (rhEPO) pre-treatment and post-treatment on isoproterenol (ISO) induced changes in Wistar rats.

Values are mean ± SE (n = 3 each); Pre = 10 days pre-treatment with erythropoietin followed by isoproterenol on the next day.Post = Isoproterenol followed by erythropoietin treatment after 2 hr.The 'F' and 'P' values are by one way ANOVA with Bonferroni 't' test for multiple comparison. [a]Significantly different from control.[b]Significantly different from isoproterenol.[c]Significantly different from erythropoietin pre-treatment + isoproterenol.

The mean values of collagen I positive cells in control, isoproterenol, pre-administration of erythropoietin (10 days) + isoproterenol, and isoproterenol followed by erythropoietin administration (2 hrs after ISO) are 18.83, 66.53, 50.97 and 12.10, respectively.Compared to the control group, the isoproterenol group and pre-administration of erythropoietin + isoproterenol groups showed 3.5 and 2.7-fold increases in the cells (P < 0.001 and 0.009, respectively). The isoproterenol followed by the erythropoietin administration group showed no change (P = 1.0). This shows that the pre-administration of erythropoietin has no effect, and post-treatment has a significant beneficial effect.The mean values of caspase I positive cells % in control, isoproterenol, pre-administration of erythropoietin (10 days) + isoproterenol, and isoproterenol followed by erythropoietin administration (2 hrs after ISO) are 23.5, 70.77, 48.70 and 22.57, respectively. Compared to the control group, the isoproterenol group and pre-administration of erythropoietin + isoproterenol group showed a 3.0 and 2.07-fold increases in the caspase-1 positive cells (P < 0.001 and 0.001, respectively). The isoproterenol followed by erythropoietin post-administration group showed no change compared to the control group (P = 1.0). This shows that erythropoietin post-treatment has significantly beneficial effects compared with pretreatment.The mean values of p-smad3 positive cells % in control, isoproterenol, pre-administration of erythropoietin (10 days) + isoproterenol, and isoproterenol followed by erythropoietin administration (2 hrs after ISO) are 5.77, 79.00, 59.70 and 22.40 respectively. Compared to the control group, the isoproterenol group, pre-administration of erythropoietin + isoproterenol group showed 13.69 and 10.35 increases in the p-smad3 positive cells (P < 0.001, 0.001, and 0.054 respectively). The isoproterenol followed by erythropoietin post-administration group showed a 3.88-fold increase compared to the control group (P <0.054), but it was not statistically significant. This shows that post-treatment with rhEPO has a better protective effect than the pre-administration of erythropoietin.The mean values of ASC positive cells % in control, isoproterenol, pre-administration of erythropoietin (10 days) + isoproterenol, and isoproterenol followed by erythropoietin post administration (2 hrs after ISO) are 0.803, 72.633, 44.567 and 21.967 respectively. Compared to the control group, the isoproterenol group, pre-administration of erythropoietin + isoproterenol group and the isoproterenol followed by erythropoietin post-administration group showed 90.45,55.50 and 27.35-fold increase in the ASC positive cells (P < 0.001, 0.017and 0.447 respectively). This shows that post-treatment with rhEPO has a better protective effect than the pre-administration of erythropoietin.

4. DISCUSSION

Histopathologicalresults of the heart tissue indicate that ISO-induced MI rats suffered the loss of myofibrillar tissue,[16] necrosis,[17] and inflammatory cell infiltration[18] as evidenced by H&E staining. As usual, the cardiac tissues of normal control rats revealed normal myofibrillar structure, cardiac architecture, and myocytes. There was an absence of edema, inflammation, and inflammatory cell infiltration. However, the heart tissue of rhEPO-pretreated rats showed considerably reduced damage with reduced myofibrillar loss, whereas, in hEPO post-treated rats, very less inflammatory infiltration was noticed. Therefore, the protective effect of hEPO pre-treatment in preventing the ISO-induced MI was fairly better than that of post-treatment with hEPO. The loss of cardiomyocytes (histomorphometric data) observed in ISO-induced rats were reversed by EPO pre and post-therapy, implicating their efficacy in reducing myocardial damage, and this effect was found to be pronounced in EPO post-therapy. Evidence from animal studies indicates that EPO treatment during ischemia/reperfusion in the heart helps to reduce the infarct size and the level of apoptosis. The beneficial effects of Epo on the endothelium include anti-apoptotic, mitogenic, and angiogenic function.[19] EPO has been known to exert a therapeutic effect in an experimental model of cardiovascular disorders, either by ameliorating apoptosis of cardiac myocytes, smooth muscle cells, and endothelial cells or by increasing nitric oxide production through the endothelial.[19] EPO, apart from stimulating the mobilization of progenitor cells from bone marrow, seems to improve the neovascularization to promote the repair of damaged endothelium[20] to render cardiac protection through a signal transduction pathway involving EPOR-β-common heteroreceptor that leads to activation of Jak2 to stimulate PI3K/Akt, NFκB, MAPK signaling pathways. Taken altogether, EPO renders cardiac protection via increased angiogenesis and attenuation of interstitial fibrosis.The reduced expression of caspase-1 in both pre and post-treated EPO rats subjected to MI induction indicated the anti-apoptotic role of EPO and cardioprotective functions. It was shown that recombinant human Erythropoietin protects myocardial cells from apoptosis via the Janus-Activated Kinase 2/Signal Transducer and Activator of Transcription 5 Pathway in epilepsy rat model[21]. According to Moon et al, [22] the effect of a single dose of recombinant human EPO (rhEPO) on left ventricular (LV) size and function was assessed in rats after the induction of myocardial infarction (MI) by permanent ligation of the left descending coronary artery. The results indicate that after an i.p. injection of 3,000 units/kg of rhEPO immediately after the coronary artery ligation, a 50% reduction of apoptosis in the myocardial area was observed after 24h. Early studiessupport that a single high-dose injection of EPO (1,000–5,000 U/kg) administered either 12–24 h before ischemia-reperfusion (I/R)[23,24] or at the onset of ischemia[25] or immediately after reperfusion is capable of reducing the apoptosis to cardiomyocyte to prevent cardiac dysfunction. A marked reduction in the infarct size and cardiomyocyte apoptosis has also been reported following EPO intervention.[26,27,28] In the present work, we have tested the efficacy of hEPO as a single high dose post-treatment as well as pretreatment for 10 days before the induction of MI in rats. While both treatments have shown protective effects, the efficacy of a single high dose post-treatment was higher. The role of transforming Growth Factor (TGF)-β has been attributed to myocardial injury, repair, and fibrosis via the activation of both Smad-dependent and non-Smad pathways. In the myocardium, which is infarcted, TGF-β/Smadsignaling activation in macrophages seems to regulate the functions of repair and remodel. The TGF-β stimulation is associated with a response from monocytes and macrophages followed by activation of the Smad3 pathway in sites of injury.[29]Studies indicate that transforming growth factor-β1 (TGF-β1)/Smadssignaling pathways have a major role in the pathogenesis of post-infarction remodelling.[30] Following TGF-β receptor activation, Smad2 and Smad3 proteins phosphorylation occurs, forming heteromeric complexes with Smad4. Later on, nuclear translocation of these complexes takes place, which helps in the regulation/control of genes involved in fibrosis.[30,31] It has been reported that Smad3 phosphorylation is elevated in fibrotic cardiac tissues, and interestingly, the inhibitor of Smad3 reduced the profibrotic effect in mice that suffered myocardial infarction [32]. Several drugs have been tested so far in different models of myocardial infarction that have been shown to render protective effects and preserve cardiac function through inhibition of TGF-β1/Smad3 and NF-κBsignaling pathways and amelioration of myocardial inflammation and fibrosis.[33-37] To our knowledge, the present study is the first to reveal how pre and post-treatment with EPO attenuated MI via suppression of psmad3 expression. Ghoneim et al.,[38] explored the protective effect of *Adansoniadigitata* in a rat model of isoproterenol-induced myocardial injury. It was found that the levels of several cardiac marker enzymes [creatine kinase MB (CK- MB), lactate dehydrogenase (LDH), and aspartate aminotransferase (AST), IL-1ß, MCP-1, MPO, Collagen, and galectin-3) increased following isoproterenol induction. A study that involved an atrial fibrillation (AF) model in dogs revealed increased expression of collagen1, implicating left atrial (LA) fibrosis and LA-remodelling.[39] The increased PDGFR-α, STAT3, and phosphorylated-STAT3 expression, apart from altered collagen-1 and fibronectin-1 protein secretion, occurred due to PDGF-AB stimulation of LA fibroblasts in this LA model. While collagen-1 expression increased in the border regions of deteriorated CREG+/- mice with cardiac fibrosis, post-treatment with Cellular repressor of E1A-stimulated genes (CREG) recombinant protein significantly improved cardiac function by inhibiting fibrosis and by reducing the expressions of αSMA and collagen-1 in the CREG+/- mice.[40] In the present investigations, the significantly decreased collagen-1 expression seen only in the heart tissues of EPO post-treated rats following MI suggests better cardioprotective efficacy of EPO post-therapy than the pre-therapy. During myocardial infarction, ischemia causes up-regulation of pro-fibrotic TGF-β that ultimately increases collagen1-A1/A3 mRNA expression[41].The therapeutic drug pinocembrin was able to reduce the expression of NLRP3, caspase 1, and IL-1β apart from reducing fibrosis area, fibrosis-related protein collagen I, α-SMA, and TGF-β thereby decreasing atrial fibrillation in isoproterenol-induced rats.[42] Evidence favors the protective effects of EPO on myocardium via inhibition of apoptotic marker caspase-12 expression.[43] In the present investigations, the EPO post-therapy was better than EPO pre-therapy in reducing collagen1 and caspase-1 expression in the MI-induced heart tissues, implicating its efficient cardioprotective functionality. According to Pan *et al,*[44] the drug triptolide showed an important role in interrupting the activation of the NLRP3 inflammasome to reduce cardiac fibrosis in isoproterenol-induced mice and especially through suppression of NLRP3 and apoptosis-associated speck-like protein (ASC). Similarly, the EPO therapy also rendered cardioprotective effects in ISO-induced rats by suppressing apoptosis-associated speck-like protein expression. However, the effect of EPO post-treatment was better than the pre-therapy. Earlier work[14] with the same experimental design (both ISO induction and rhEPO treatments) suggested that both pre and post-therapy of rhEPO in ISO-induced MI have beneficial effects. Based on their results and findings, both pre-(injecting the rats with a daily dose of rhEPO for 10 days before MI) and post-therapy with rhEPO (a single dose of rhEPO 2 hours after MI) revealed a significant decrease in infarction size and plasma level of cardiac enzymes (CPK: Creatine phosphokinase and LDH: Lactate dehydrogenase). However, the present work explored the histological and immunohistochemical alterations in the heart tissue to understand the therapeutic benefits of rhEPO in the MI model.

5. CONCLUSIONS

While EPO has been used as a therapeutic agent to ameliorate myocardial infarction and associated complications, the present study aimed to explore and compare the efficacy of EPO pre and post-therapy on histomorphological and immunohistochemical changes in ISO-induced MI rats. The study finding concludes that EPO post-therapy after MI had better therapeutic efficacy in ameliorating cardiac damage and fibrosis via altering key proteins associated with cardiac damage/repair. Although ten days' pre-treatment with EPO followed by MI revealed therapeutic benefits, better efficacy was noticed with EPO post-therapy. Therefore, the present animal study concludes that EPO post-therapy can significantly reduce the adverse complications associated with MI and improve cardiac repair.

6. AUTHORS CONTRIBUTION STATEMENT

THP and AGNS conceived the idea and designed the experiments. THP and SS performed experiments. RV analyzed the data. THP and SS wrote the manuscript. RV and SS edited and revised the manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declarednone.

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