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ORIGINAL ARTICLE

Role of Fingolimod in Attenuation of Lymphocyte Count Leading to Reduced Apoptosis in Myocardial Ischemia Reperfusion Injury

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ABSTRACT

Background: FTY720 (Fingolimod) is a drug having immune-regulatory properties. It is a structurally analogous to Sphingosine-1-phosphate. S1P is a biologically active lipid mediator in various inflammatory pathways.
Aim: To investigate the effects of FTY720 on myocardial ischemia reperfusion (IR) injury in cardiac surgery.
Study design: Experimental study
Place and duration of study: Aga Khan University, Karachi and Khyber Medical University, Peshawar Pakistan from 1st January 2017 to 31st December 2020 with collaboration of University of Verona, Italy.
Methodology: Twenty Sprague-Dawley rats were segregated into two groups; treatment and control. In treatment group received FTY720 at 1 mg/kg, intravenously 15 minutes prior to the experiment. Both groups were exposed to myocardial ischemia (30 m) reperfusion (2 h). Blood gas analysis, lymphocyte count, myeloperoxidase assay and TUNEL assay were performed and analyzed to observe the effect of FTY720 on neutrophil infiltration and apoptosis.
Results: FTY720 treated group had improvement in blood gas levels in contrast to the control, treatment group also experienced decrease in neutrophil infiltration, lymphocyte count and myeloperoxidase enzyme expression.
Conclusions: FTY720 pre-treatment reduce lymphocyte count that leads to reduce the level of apoptosis and salvage the myocardial damage incurred by ischemia reperfusion.
Keywords: Fingolimod, Cardiopulmonary bypass, Lymphocyte, Neutrophil, Myeloperoxidase

INTRODUCTION

Myocardial reperfusion may lead to unfavourable cardiovascular consequences following myocardial ischemia, heart surgery or circulatory halt.[1] Myocardial injury is mediated by leukocyte infiltration, myocardial capillary disturbance, pulmonary epithelial cell swelling and ultimately the death of myocardial cells.[2,3] Animals and humans can be used to display well developed myocardial ischemia reperfusion injury (IRI),which leads to numerous local and systemic outcomes, resulting in multiple organ failure and finally can causedeath.[4,5] It has been found that ischemic myocardial tissues are vulnerable to stimulated neutrophils and free oxygen species.[6] Though, the pathogenesis of IRI consists of complex processes; polymorphonuclear (PMN) leukocyteis thought to be a key player in initiating myocardial injury.[7] Therefore, reduction of myocardial PMN sequestration may serve as a therapeutic target to reduce myocardial ischemia reperfusion injury. Sphingosine-1-phosphate (S1P) is a lysophospholipid, derived from sphingosine by the action of sphingosine kinase 1 and 2 (SphK2, SphK2) through several pathways; S1P lyase (S1PL) takes active part in thismetabolism.[8] It is suggested that S1P can regulate a number of biological processes. It acts as second messenger in various intracellular pathways involved in the cell proliferation and apoptosis; inflammatory cell recruitment to the site of injury and maintaining vascular endothelial integrity[9]. Moreover, some studies also suggested thatS1P is also involved in organ ischemic reperfusion injury1.0[11].

FTY720 (Fingolimod) is a structural equivalent of S1P. It is converted to FTY20-P by SphK2. This phosphorylated product FTY720-P binds to G-protein coupled S1P receptor subtypes 1, 3, 4, and 5. This binding leads to reduced lymphocytic cell migration from thymus and lymph nodes[12].

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It is suggested that Fingolimod and its derivatives can be useful and effective drug target for protection of organs from ischemic reperfusioninjury.[13-15] Inhibition of neutrophil priming is the suggested pathophysiological mechanism of this protection conferred by FTY720[16]. Fingolimod is also identified to have effective long term anti-inflammatory and antioxidant effects. It reduces myocardial fibrosis, which results in lesser cardiomyocyte death[17]. Fingolimod stimulates reperfusion injury salvage kinase (RISK) and initiates survivor activating factor enhancement (SAFE) pathways which subsequently impede the pro-apoptotic and promotes the action of anti-apoptotic pathways[18].

This research was aimed to evaluate the outcomes of FTY720 exposure on myocardial IRI related to cardioplegic arrest in rat model.

MATERIALS AND METHODS

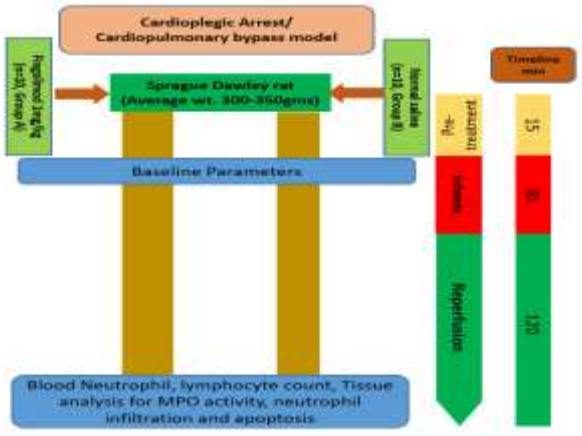
This research was done at Aga Khan University, Karachi and Khyber Medical University, Peshawar Pakistan from 1st January 2017 to 31st December 2020 with collaboration of University of Verona, Italy. The study was conducted in compliance with high quality research practices according to the Declaration of Helsinki principles and permission from IRB. The study was given approval by the local Joint Ethical Committee for University of Verona and Hospitals (Verona and Rovigo), Italy (BBCCH1337) and institutional animal care committee.

Twenty male Sprague–Dawley rats having weight between 300–350 g, were arranged from Harlan Laboratories (Udine, Italy). The rats had free access to standard rat chow diet. Around 3–4 rats were placed per cage, exposed to 12-h light/dark cycle and temperature was kept at 21°C. They were placed on the heating board in supine position to maintain the rectal temperature of 37°C during the surgery before cardioplegic arrest (CPA). For anaesthesia, sodium pentobarbital was used at 30 mg/kg intraperitoneally. Intubation was done through oropharynx with 14 polyethylene tube and mechanical ventilation was given by a

rodent ventilator from Harvard Apparatus Inc., Holliston, MA. The breathing rate was 50-60/min, tidal volume was maintained at 6 ml/kg, using an air-oxygen mixture (inspired oxygen fraction = 0.5)[19]. To record the systemic arterial pressure, heparinized 24-gauge Teflon catheter was used to cannulate the left femoral artery. A modified 4-hole 16-gauge angiocath catheter was progressed into the right atrium with good drainage. An 18-gauge catheter was used to cannulate left common carotid artery in order to access the aortic arch. Total heparinization (500 IU/kg) was performed after surgical preparation and rapidly done before cardiopulmonary bypass and initiation of CPA to prevent blood from clotting in the circulation. The setup included venous reservoir, roller pump, hollow fiber oxygenator (Sorin, Mirandola, Italy), and a vacuum regulator with the applied pressure of -30 mm H_2O to assist venous drainage, all were linked with plastic tubing.

The rats were distributed into two groups; treatment and control. Treatment group was given FTY720 intravenously at 1 mg/kg, 15 minutes prior to initiation of surgical procedure. CPA was initiated in both groups by giving cold St. Thomas solution (used for the safety of the myocardium during an open heart surgery).Extra corporeal life support (ECLS) was given for the next 2 hours, aorta was clamped for initial 30 minutes of ECLS.[20] Temperature of 36-37°C was maintained in both groups throughout ECLS. After 2 hours of reperfusion, ECLS was removed in the rats of both groups. Blood samples for analyses mentioned below, were collected before starting the experiment and after the completion of reperfusion. Tissue samples were collected from myocardium and preserved at -80°C. A diagram review of experimental design is presented in Figure 1.

Fig. 1: Experimental design of study



Arterial blood gas (ABG) analysis was performed with i-STAT Portable Clinical Analyzer (i-STAT Corporation, East Windsor, New Jersey), before start of ischemia and after reperfusion. This test was used to measure partial pressure of oxygen (PaO_2), carbon dioxide ($PaCO_2$), Base excess (BE) and pH. Blood samples of control and treated group were tested for total and differential cell counts at baseline and then at completion of reperfusion.

For colorimetric studies mostly MPO assay is used. Myeloperoxidase (MPO) is a heme based enzyme and present in abundance within neutrophils. It is spectrophotometrically considered as an indicator of neutrophil infiltration in ischemic tissue. Samples from earlier preserved myocardial tissues from all groups were thawed and were added in 4mL buffered solution of pH 5 containing 50mmol of potassium phosphate and 0.5% hexadecyltrimethyl ammonium bromide (Sigma) for 2 minutes. Then, each sample was mixed for 1 minute and centrifuged at 15,000RPMat 4°C for 15 minutes. The supernatant of samples were saved and treated with 100mm hydrogen peroxide/sodium acetate and tetramethylbenzidine solution for 2 minutes. The difference of absorbance over 1min was calculated by spectrophotometer at 655nm. One unit MPO activity was considered as the number of enzyme degrading 1µmol of peroxide in one minute at 25°C for each gram of the tissue[15].

After the completion of reperfusion, myocardial samples were taken for histological assays of polymorphonuclear leukocytes (PMN). Myocardial samples saved at -80°C were thawed, mixed and stained with naphthol AS-D chloroacetate (specific esterase). PMN positive staining was used to check if any neutrophil was present. Blind examination was done by trained pathologist and counting of PMN was done under light microscope with 400x magnification.

Click-iT Plus terminal deoxynucleotidyl transferase (dUTP) nick end labelling (TUNEL) assay was performed to recognize in Situ Apoptosis. 100% xylene was used to deparaffinize the myocardial tissues obtained from both groups and then serial dilutions with ethanol were prepared. Apoptotic cells were stained with TUNEL assay. The reorganization was done by green fluorescent reagent called Alexa Fluor 488 dye obtained from Molecular Probes Life Technology (Thermo Scientific). Nuclei were stained with Hoechst 33342 solution and observed.

SPSS version 21 was used to analyze the data. Comparison was made between the two groups by student's t-test or its nonparametric alternative Mann–Whitney U test wherever applicable. Level of significance was taken at p value of <0.05.

RESULTS

As compared to baseline, control group has greater worsening of the pH, PaO_2, $PaCO_2$ and BE at 2 h of reperfusion. Fingolimod resulted in an improved blood gas profile in this model. The comparison of treatment group was done with baseline and control group. Results of both comparisons were statistically significant (p<0.05) [Table 1].

The results of cell count displayed that Fingolimod resulted in a reduction in percentage of circulating lymphocytes (54.48±1.82%) and total white cell count (4589.74±293.94) 2 h after injection (P<0.001) compared to the control (82±0.781%, 18008.88 ± 850.327) and baseline (81.2±0.238, 18629±940.349) groups (Fig. 2).

Colorimetric MPO assay showed the decreased tendency of neutrophil's movement to the injury site in treated group. Concentration of myeloperoxidase was noticeably elevated in the control group. MPO is found in the neutrophil granules and can be used to measure neutrophil activity in a tissue. From above findings, it can be concluded that administration of FTY720 significantly decreases the migration of neutrophils to the myocardial tissue post reperfusion. Reduction in PMN count in treated group also favours this concept. Comprehensive observation and analysis showed that PMN count was decreased from (2 PMN)/50 high-power fields (HPF)] in control group to (1 PMN)/50 high-power fields (HPF)] in treated group (Fig. 3).

TUNEL Assay's results showed extensive myocyte apoptosis in control group than in the group treated with FTY720. Fluorescence stereomicroscope at 20x magnification showed the programmed cell death in both groups. The assay provides some information about cell apoptosis and showed relative differences in the rate of programmed cell death between controlled and treated groups. Slides D, E and F show apoptosis after 2 h of reperfusion in the control group compared to negative control slide 3A-C. Apoptotic nuclei expression was greatly reduced in Fingolimod treated group (Fig. 4).

Table 1: Effect of Fingolimod on blood gases

Variables	Baseline	Control	Fingolimod treated
pH	7.35±0.14	7.21±0.12*	7.32±0.16#
PaO_2 (mmHg)	249±47	148±52*	253±58#
$PaCO_2$ (mmHg)	43±7	66±12*	51±7#
BE (mmHg)	-3.1±1.3	-7.3±2.1*	-4.1±1.5#

*Significant (P<0.05) for comparison of Baseline and Control group, #Comparison of treatment vs control group (p<0.05)

DISCUSSION

Fingolimod (FTY720) is a structural analogue of S1Pand is approved by FDA for preventing relapses in multiple sclerosis.[21,22] In addition to a potent immune-modulating effect, Fngolimod also has anti-apoptotic, anti-inflammatory and anti-oxidative activities.[23]

Sphingosine is phosphorylated by SphK1 and SphK2 into Sphingosine 1-phosphate. Interaction between S1P and its receptors initiates a series of immunological pathways, which effectly mphocyte translocation[24], cytokine cycle of inflammation[25] and apoptosis.[26] SphK1 plays an important role in regulating inflammatory processes, and can be over stimulated in many disease conditions. FTY720 is a prodrug and requires phosphorylation into FTY720-p by SphK2 for effective interaction with S1P receptors.[27,28] One study found that theS1P can lead to greater myocardial resistance, and formation of proinflammatory cytokines in mice in a dose dependent manner, when delivered systemically.[29] Inhalational administration of FTY720 and SphK inhibitors reduced asthma exacerbations, reinforcing its anti-inflammatory role[30].

Fig. 2: Pre-treatment with Fingolimod considerably altered percentage of circulating lymphocytes and neutrophils in comparison to baseline and control groups. *p<0.05, **p<0.001

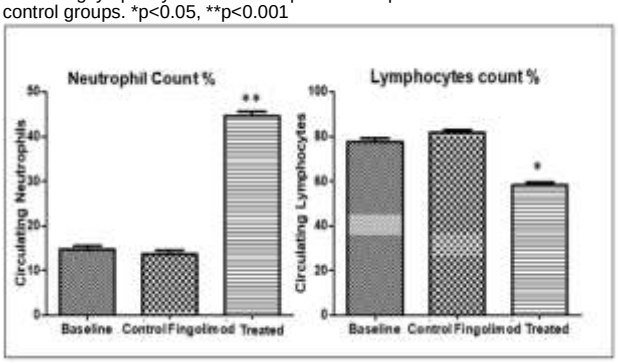


Fig. 3: Comparison of myeloperoxidase levels and the number of tissue neutrophils in rat myocardium after ischemia- reperfusion, *p < 0.05, **p < 0.001.

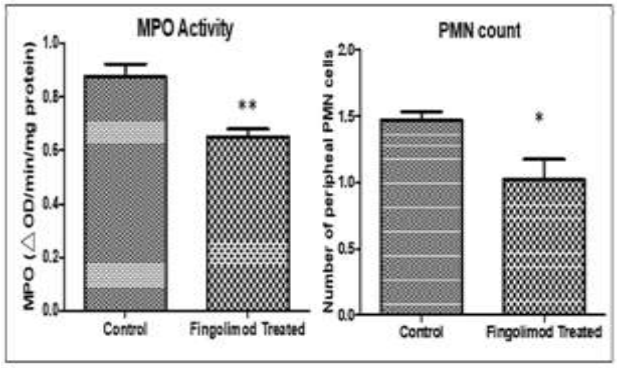
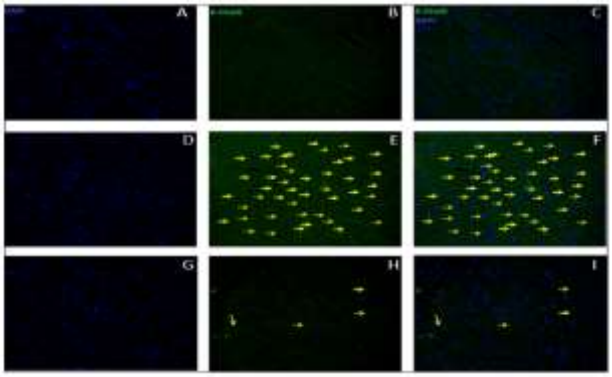


Fig. 4: Photomicrographic presentation of TUNEL-positive nuclei in the groups. A, B and C are images of negative control, D, E and F are the images of control group; G, H and I are the images of treated group



Previous studies had proved that premedication with S1P modulator protected the myocardial tissue from IRI.[31] Occlusion of the arterial supply to myocardium incurs variable degrees of ischemic injury. In addition, the reperfusion of this ischemic tissue may cause necrosis, microcirculatory disruption leading to further myocardial damage.[32] During this process, activated neutrophils in the myocardial tissue stimulate the generation of free radicals, proteases, and pro inflammatory cytokines.[33] Various studies proposed this IR also exacerbate cardiac impairment and myocyte injury, heralding inflammation and apoptosis of already ischemic cardiomyocytes.[34] TUNEL method is the standard assay used to recognize the level of apoptosis.[35]

In the present study, we evaluated the pre-treatment effect of Fngolimod on myocardial damage and indicated that FTY720-P may provide protection in improving myocardial injury through improved oxygenation, preventing migration of neutrophils, reduced apoptosis. Our study found that FTY720 preconditioning significantly reduced the myocardial damage and neutrophilic infiltration of the tissue. Our study results are also supported by different previous studies, which suggested the protective effect of FTY720.[36-38] One study stated that FTY720 limited the exit of lymphocytes from lymph nodes, thus reducing the numbers of circulating lymphocytes.[39] A study assessing the effect of Fingolimod in the mouse kidney subject to ischemic reperfusion injury also noted similar changes.[40] When FTY720 was given to cats, it decreased the circulating neutrophil counts.[41] This is in contrast to our findings. Another work on mouse model of liver IRI demonstrated that reduction of CD4 T lymphocytes and hepatic neutrophil permeation resulted in improved hepatic function.[42] All these findings strengthen the idea that tissue penetration of neutrophils during IR may be a lymphocyte-driven process; FTY720 can reduce this neutrophilic infiltration mitigating the ischemic reperfusion injury.

One of the study found that FTY720 improved myocardial recovery in animals as an immunomodulatory agent with activation of S1P receptors.[43] Inflammation is the major factor of ischemic reperfusion injury. IL-6, and TNF-α are the major pro-inflammatory cytokines contributing to myocardial impairment.[44-46] The protective effect of Fingolimod is evident from our experiment. Our study evaluated that blood gas levels were improved in Fingolimod group in comparison to that of control. Treatment group showed immunomodulatory effect by decreased number of lymphocytes and a lower expression of myeloperoxidase enzyme. Treatment with Fingolimod also decreased apoptosis in the ischemic reperfusion model, as supported by the literature.[43,47] This reduction in apoptosis occurred due to RISK and SAFE pathways.[43,48] In our experiment, the control showed a higher level of apoptosis while FTY720 treated rats showed lower apoptosis. The results from TUNEL assay proved that activating anti-apoptotic cascade is also one of the possible mechanisms of cardio protection by FTY720. In this study, administration of Fingolimod resulted in reticence of apoptosis and myocardial damage.

CONCLUSION

In conclusion, FTY720 pre-treatment reduce lymphocyte count that leads to reduce the level of apoptosis and salvage the myocardial damage incurred by ischemia reperfusion.

Conflict of interest: Nil

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