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Comparative evaluation of HMG CoA reductase inhibitors in experimentally-induced myocardial necrosis: Biochemical, morphological and histological studies

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ABSTRACT

The present study was carried out to evaluate the protective effect of different statins on isoproterenol (ISO) induced myocardial necrosis. Atorvastatin, rosuvastatin, fluvastatin, simvastatin and pravastatin (10 mg/kg/day) were administered for 12 weeks. After pretreatment of 12 weeks myocardial necrosis was induced by subsequent injection of ISO (85 mg/kg/day, *s.c.*) to wistar rats. Serum biochemical parameters like glucose, lipid profile, cardiac markers and transaminases were evaluated. Animals were killed and heart was excised for histopathology and antioxidant study. Statins pretreated rats showed significant protection against ISO induced elevation in serum biochemical parameters and serum level of cardiac marker enzymes and transaminase level as compared to ISO control group. Mild to moderate protection was observed in different statins treated heart in histopathology and TTC stained sections. Result from our study also revealed that statins could efficiently protect against ISO intoxicated myocardial necrosis by impairing membrane bound enzyme integrity and endogenous antioxidant enzyme levels. Amongst all statins used, rosuvastatin and pravastatin were found to have maximum cardio-protective activity against ISO induced myocardial necrosis as compared to other statins.

1. Introduction

According to WHO, cardiac disorders are the second leading cause of death with an estimate of 7.8 million deaths in 2013 and it has been predicted that the number of deaths will tremendously increase to 9 million by the year 2025 (Baldwin et al., 2013). Myocardial necrosis results from prolonged ischemia to the cardiac myocytes due to interruption of the blood flow to the particular part of the heart, deposition of fat in coronary arteries, deprivation of body anti-oxidant mechanisms, elevated production of free radicals which ultimately aggravate cardiovascular complications like arrhythmia, cardiomyopathy, angina pectoris, congestive heart failure etc. (Das and Maulik, 1995; Whellan, 2005).

In 1976, Endo et al. discovered statins as fungal metabolites having inhibitory action on 3-hydroxy 3-methylglutaryl co-enzyme A (HMG CoA) reductase, a rate limiting enzyme in trail of *de novo* cholesterol synthesis, resulted into hypocholesteromic activity (Endo et al., 1976). In addition to this, all statins possess additional pleiotropic actions independent to their lipid lowering action, like improvement of endothelial dysfunction (Marchesi et al., 2000; Murata et al., 2005), anti-inflammatory (Antonopoulos et al., 2012; Blake and Ridker, 2000; Lefer, 2002), anti-oxidative (Adam and Laufs, 2010; Buyukhatıpoglu et al., 2010; Parihar et al., 2012), anti-thrombotic (Ferrara et al., 2003; Mitsios et al., 2010; Violi et al., 2013), cytoprotective (Vítek and Lenicek, 2006), anti-proliferative (Riganti et al., 2011; Vítek and Lenicek, 2006; Wali and Sylvester, 2007), neoangiogenic effect (Krum and McMurray, 2002). However, effect of these statins, on changes in endogenous antioxidant system during myocardial necrosis, remain to be elucidated. In the present study, ISO induced myocardial necrosis model was used for assessment and comparison of ameliorative action of various statins by correlation of various biochemical, morphological and histo-architechtural changes.

2. Materials and methods

2.1. Drugs and reagents

Atorvastatin, rosuvastatin, fluvastatin, simvastatin and pravastatin were obtained as a gift sample from Alembic Pharmaceutics, Baroda

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and Torrent Research Center, Bhatt. Isoproterenol hydrochloride (ISO) and 2,3,5-Triphenyl-tetrazolium chloride (TTC) were purchased from Sigma Aldrich, Co. St. Louis, MO, USA. All other chemicals and reagents used in study were of analytical grade and procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Kits for the estimation of glucose, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), creatine phosphokinase-MB (CK-MB), total cholesterol and triglycerides, were obtained from Span diagnostics Pvt. Ltd., India and Reckon Diagnostics Ltd. India.

2.2. Experimental animals

Male wistar rats weighing 300–325 g were used and experimental protocols described in the present study were approved by the Institutional Animal Ethics Committee of Pharmacy Department, Faculty of Technology and engineering, The M. S. University of Baroda, Vadodara. All experimental procedures were in accordance with CPCSEA guidelines, Ministry of Environment and Forests, Govt. of India. The animals were housed in controlled conditions (12 h light and dark cycle at 20–25 °C with 35–65% relative humidity). Paddy husk was used as bedding material and changed on alternate days. Animals were provided with pelleted food (Pranav Agro Foods Pvt. Ltd., Vadodara) and purified water *ad libitum*.

2.3. Experimental design and protocol

All animals were randomly allocated into 7 different groups with 6 rats in each group. Group-I was normal control group and group-II was disease control group treated with equal volume of 0.5% CMC solution. Group-III to VII rats received atorvastatin (ATO), rosuvastatin (ROS), fluvastatin (FLUVA), simvastatin (SIMVA) and pravastatin (PRAVA) respectively at evening time (between 20.00 and 22.00 h; 10 mg/kg/day, *p.o.*) for 12 weeks. Although, all mentioned statins have different pharmacodynemic and pharmacokinetic profile, same dose was chosen to compare the cardioprotective efficacy of the statins. At the end of 12 weeks of statins administration, myocardial necrosis was induced by two injections of ISO (85 mg/kg, *s.c.*) on the penultimate day and ultimate day of the treatment. All the animals from group II–VII received ISO while animals in group I was administered with an equivalent volume of saline. Blood was withdrawn one hour after last ISO administration by retro-orbital puncture and serum was separated and stored at −20 °C till further biochemical analysis. Serum samples were analyzed for estimation of blood glucose, total cholesterol, HDL-cholesterol, AST, ALT, LDH and CK-MB levels. LDL-cholesterol level was calculated according to Friedewald's formula (Friedewald et al., 1972) and atherogenic index was calculated as ratio of non-HDL cholesterol to HDL cholesterol (Kayamori and Igarashi, 1994). After collection of blood samples, animals were killed and heart was excised, rinsed with ice-cold phosphate buffered saline (pH 7.4) and weighed for estimation of myocardial burden related to edematous and fibrous tissue. Heart weight to body weight ratio was calculated as described earlier (Ramesh et al., 1998). Ventricles were harvested, cut and aliquoted into two separate tubes. One aliquot was stored in 10% v/v neutral buffered formalin for histoarchitecture studies. Second aliquot was homogenized in phosphate buffered saline (pH 7.4) for determination of oxidative stress related parameters and estimation of various membrane bound ATPase enzymes. Total protein content was determined as per the method previously described by Lowry et al. 1951.

2.4. Assessment of oxidative stress-related markers in heart

Tissue homogenate was mixed with equal amount of TCA solution (10% w/v), after centrifugation clear supernatant was used for determination of malonaldehyde (MDA) according to method described previously (Slater and Sawyer, 1971). Supernatant was mixed and boiled with identical volume of TBA solution (0.67% w/v) and after cooling for 5 min, absorbance was measured at 532 nm. The results were expressed in terms of nM of MDA/mg of protein. Glutathione (GSH) was measured by the method described elsewhere (Moron et al., 1979) and the results were expressed in terms of μg of GSH/mg of protein. Catalase and superoxide dismutase (SOD) were analyzed by kinetic absorption method prescribed previously by Aebi (Aebi, 1984) and Misra (Misra and Fridovich, 1972) respectively. Catalytic activity was expressed for catalase as μmoles of H_2O_2 consumed/min/mg of protein and for SOD was expressed as units/min/mg of protein.

2.5. Estimation of membrane bound ATPases

The residue of heart homogenate was resuspended in PBS (pH 7.4) and same solution was utilized for estimation of various membrane bound ATPase enzymes.

2.5.1. Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity was measured according to the method described previously (Bonting, 1970). Briefly, reaction was initiated by adding resuspended solution into reaction buffer containing 0.2 ml each of 5 mM $MgSO_4$, 5 mM KCl, 60 mM NaCl, 0.1 mM EDTA, 40 mM ATP and 1 ml of 92 mM Tris–HCl buffer (pH 7.5). After incubation for 30 min at 37 °C, the reaction was arrested by addition of ice cold 10% TCA and solution was centrifuged at 1000*g*, the supernatant was used for the estimation of inorganic phosphorus (P_i) using method described by Fiske and Subbarow (Cyrus and Subbarow, 1925). The enzymatic activity was expressed as μmoles of P_i liberated/mg protein/h.

2.5.2. Mg^{++}-ATPase activity

Mg^{++}-ATPase activity was assessed using the method described formerly (Ohnishi et al., 1982). Resuspended solution was mixed with equal volume of 374 mM Tris–HCl buffer (pH 7.6), 25 mM $MgCl_2$ and 10 mM ATP. The resultant reaction mixture was allowed to incubate for 15 min at 37 °C and the reaction was stopped by adding 10% TCA. The resulting mixture was centrifuged at 1000*g* and supernatant was used for estimation of the liberated P_i. The enzyme activity was articulated as μM of P_i liberated/mg protein/min.

2.5.3. Ca^{++}-ATPase activity

Ca^{++}-ATPase enzymatic activity was assayed by the previously described method (Hjerten and Pan, 1983). To reaction mixture (equal volume of 125 mM Tris–HCl buffer (pH 7.5), 50 mM $CaCl_2$ and 10 mM ATP), resuspeded solution was added and the reaction mixture is incubated for 15 min. The reaction was detained by the addition of 10% TCA to the mixture and centrifuged at 1800*g* for 5 min to separate the supernatant for the estimation of P_i. Enzyme activity was expressed as μM of P_i liberated/mg protein/min.

2.6. TTC staining for macroscopic measurement of infarcted area

Macroscopic enzyme mapping of infarcted myocardium was carried out by TTC staining (Khalil et al., 2006; Ramkissoon, 1966). The excised heart was rapidly washed with ice cold buffer to remove blood content and was allowed to get frozen for 4–6 h at −20 °C. The frozen heart was cut transversely across the left ventricle to get sections not more than 0.2 mm and were placed in darkened glass plate containing 1% TTC solution in phosphate buffer. The glass plate was incubated at 37°–40 °C for approx. 30 min and sections were turned over so that they remained