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Research Article

CARDIOPROTECTIVE EFFECT OF CLOPIDOGREL BY ATTENUATING INFLAMMATION, APOPTOSIS IN ISOPROTERENOL INDUCED MYOCARDIAL INFARCTION RAT MODEL

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ABSTRACT

The present study was aimed to evaluate the cardioprotective potential of clopidogrel against isoproterenol induced Myocardial Infarction (MI) in rats. Rats were pretreated with Clopidogrel (25 mg/kg/day, p.o) for a period of 14 days. ISO (85mg/kg, s.c) was injected on 13th and 14th day of the treatment to induce MI. rats pretreated with clopidogrel significantly diminished the myocardial enzymes including creatine kinase (CK-MB), lactate dehydrogenase (LDH) and cardiac troponin I (cTnI). Decrease in ST segment elevation as well as amelioration in left ventricular dysfunction were also found in clopidogrel-treated groups. In addition, it also prevented alteration in the levels of electrolytes. Treatment with clopidogrel remarkably inhibited the protein level of caspase-3 in infarcted rats by ELISA analysis. Furthermore, clopidogrel exhibited down regulation in mRNA of IL-6, *TNF-α* and histopathological changes. The finding suggests that the cardioprotection of clopidogrel associate with its anti-inflammatory and anti-apoptotic properties in myocardial infarction of rats.

INTRODUCTION

Myocardial infarction (MI) occurs when there is myocardial necrosis due to prolonged imbalance between the myocardial oxygen supply and demand of the myocardium (Lopez and Murray, 1998). Although clinical care is improved, public awareness is raised and health innovations are widely used, myocardial infarction remains the leading cause of death worldwide (Whellan, 2005; Aronow, 2006). By 2020, heart disease and stroke will become the leading causes of both death and disability worldwide, with the number of fatalities projected to increase to more than 20 million a year, and to more than 24 million a year by 2030. Developing countries like India are struggling to manage the impact of infectious diseases simultaneously with the growing burden on society and health systems caused by noncommunicable diseases such as myocardial infarction. In India, myocardial infarction typically occurs 10–15 years earlier than in Western countries.

Oxidative stress is an important pathogenic event in a variety of cardiovascular diseases such as atherosclerosis, hypertension, ischemic heart disease and heart failure (Dhalla *et al.*, 2000; Mehany *et al.*, 2013). It occurs when excessive generation of reactive oxygen species (ROS) cannot adequately neutralized by endogenous antioxidants leading to cell membrane injury and cell death (Takimoto and Kass, 2007; Galaly *et al.*, 2014). ROS generation in ischemic tissue, bringing about oxidative damage of membrane lipids, proteins, carbohydrates, and DNA (Dikshit *et al.*, 1992). Energy depletion of the cells, necrotic type cell death along with cardiomyocyte apoptosis were also found due to oxidative stress (Radons *et al.*, 1994; Wollert and Drexler, 2002).

Catecholamines have a key role in normal cardiac function. However, Isoproterenol (ISO), a synthetic catecholamine, liberates ROS and causes as infarct like necrosis of the myocardium resembling MI in humans at toxic doses(Nagoor Meeran *et al.*, 2012). MI evokes intense inflammatory responses both systemically and within the infracted myocardium, with adverse consequences. The potential contribution of platelets to post infarct cardiac inflammation remains unexplored. Relevant to this is the question of whether Clopidogrel exerts cardiac protection through inhibition of platelet's inflammatory action in the infracted myocardium, independent of vascular thrombosis is remaining unexplored. Also, It was reported recently that Clopidogrel has anti-inflammatory, antioxidant and anti-apoptotic activity (Hu *et al.*, 2011). Clopidogrel may exerts its beneficial effect by either necrotic pathway or apoptotic pathway. Both are deeply involved in myocardial ischemic damage. However, its role in prevention of myocardial damage and the mechanism is unknown.

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Thus, the present study was carried out to find out the cardioprotective effect of Clopidogrel in ISO induced MI by exhibiting anti-apoptotic and inflammatory action. To investigate this cardiac marker enzymes, haemodynamic alteration, lipid peroxidation levels, caspase-3 protease level along with gene expression of IL-6 and *TNF-α* by RTq-PCR were investigated in ISO induced myocardial infarction in rats.

MATERIALS AND METHODS

Drugs and preparation of solutions

Clopidogrel was obtained as gift sample from Alembic Pharmaceuticals Ltd., Vadodara. Clopidogrel was freshly prepared everyday by dissolving it in a 0.5% CMC suspension.

Dose fixation study

Pilot study was performed for the dose fixation of Clopidogrel. Three doses (10 mg/kg, 25 mg/kg and 50 mg/kg) were evaluated in the project. Most efficacious dose (25 mg/kg) was incorporated in the final protocol.

Chemicals and kits

All the chemicals used in this project were of analytical grade and were obtained from Astron chemicals, Ahmedabad, and SD fine chemicals, Mumbai, India. Isoproterenol (Thiry *et al.*) hydrochloride was purchased from Sigma Chemical Co. A (St Louis, MO, USA). All the biochemical tests were performed using the standard reagent kits purchased from CPC Diagnostics, Chennai. Troponin-I (Cal-Bio company) and Caspase-3 (Bio-Rad company, model 680XR) level was analysed using ELISA kit. For gene expression study: RNA later (Ambion, Inc), TRI (Sigma), DNase I (Qiagen), First-strand cDNA synthesis (Thermo scientific) and SYBR Green PCR kit (KAPA) were used. The primers were first designed by using NCBI BLAST primer tool and then commercially synthesized (Eurofins Genomics).

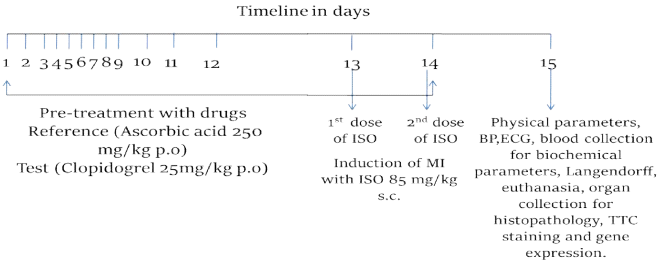
Animals

Healthy male Wistar rats weighing 250 ± 20 g were used for the study. The rats were housed in a group of 6 rats per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12h/12h light-dark cycle. Animals had free access to conventional laboratory diet (purchased from Pranav Argo Pvt. Ltd) and water *ad libitum*. The protocol (APC/2014-IAEC/1410) of the experiment was approved by Institutional Animal Ethical Committee as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Isoproterenol induced Myocardial Infarction and experimental design (Gong et al., 2012*)*

Animals were randomly allocated to Four groups containing ten animals in each. Group I served as control. Myocardial infarction was induced in groups II-IV by subcutaneous administration of ISO (85mg/kg s.c.) at 24 h interval on 13th and 14thday of the study. Group II served as ISO control (model control), Group III received ascorbic acid (250 mg/kg p.o.), and group IV received Clopidogrel (25mg/kg, p.o.) once a day for 14 days.

After the 24 h from the last injection of ISO, blood pressure and ECG were recorded. Blood samples were collected retro-orbitally under anaesthetic condition with urethane (1 g/kg i.p.) and stored for 30 min. at room temperature to obtained the serum. Serum was obtained by centrifugation at 760 x g for 10 min and then it was used for the estimation of cardiac marker enzymes like CK-MB, LDH, Troponin-I and electrolyte concentration (Na^{+}, $K^{+,}$ and Ca^{+2}). Simultaneously, the heart was isolated and rinsed with ice-chilled physiologic saline and used for Langendorff and then weighed. Weighed heart was used for homogenate, histopathology, and RTq-PCR. In heart homogenate antioxidant levels were estimated.



Langendorff Isolated Perfused Heart Preparation

Rats were given intraperitoneal (i.p) injection of 500 IU heparin and anesthetized by i.p. injection of 50-80 mg/kg pentobarbital. After the rat became unconscious and lost pedal reflex activity, the heart surgery was started. A midsternal thoracotomy was performed to open its chest. Second, the heart was hastened to excise and to transfer into oxygenated ice-cold modified Krebs-Henseleit buffer. Then, the cannula filled with oxygenated modified Krebs-Henseleit buffer was tied to the aorta. Finally, the heart attached cannula was rapidly switched to connect with the Langendorff perfusion apparatus. The apparatus had a constant flow rate of the modified Krebs-Henseleit buffer saturated with carbogen (95% O2 and 5% CO2), and its temperature was controlled at 37°C. In order to measure pharmacodynamic response, a latex balloon tied to the end of a polyethylene tube, which was connected with a pressure transducer was carefully inserted into a left ventricle of the isolated heart. The balloon was inflated with 50% methanol to create a diastolic pressure of 5 to 6 mmHg. Hemodynamic parameters like left ventricular end diastolic pressure (LVEDP), heart rate (HR), coronary flow rate, $+dp/dt_{max}$ and $-dp/dt_{max}$ were recorded.

Statistical analysis

Results presented are mean ± S.E.M. Data were analysed with one-way ANOVA followed by Dunnet Post hoc test using graph pad prism 6.0 software. A value of $P<0.05$ was considered statistically significant.

RESULTS

Effect of isoproterenol induced myocardial infarction (MI) on physical parameter

The effects of Isoproterenol (85 mg/kg s.c.), Ascorbic acid (250mg/kg, p.o.) and Clopidogrel (25 mg/kg, p.o.) treatment on heart weight, tibial length and heart weight to tibial length ratio are depicted in Table 1. There was significant difference in the heart weight and tibial length between the groups

observed, isoproterenol treated animals showed a significant increase in heart weight as well as tibial length. The heart weight to tibial length ratio was increased significantly in ISO group rats when compared with control rats. In rats pre-treated with Clopidogrel and then treated with isoproterenol, there was insignificant reduction in the ratio of heart weight to tibial length as compared to isoproterenol treated rats. however, significant difference in heart weight was observed.

Table 1 Effect of isoproterenol induced myocardial infarction (MI) on physical parameter

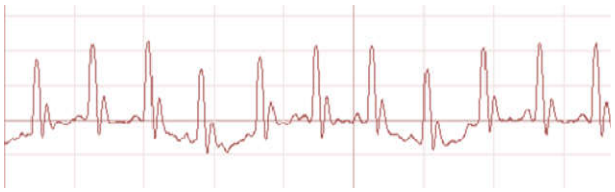
Groups	Heart weight (g)	Tibial length (cm)	Heart weight/tibial length (gm/cm)
Control	(0.76± 0.01)	(5.15± 0.10)	(0.14 ±0.005)
ISO (85 mg/kg)	(1.2 ±0.04) ####	(6.17± 0.12) ####	(0.19 ±0.006) ##
Ascorbic acid (250 mg/kg,p.o.)+ISO	(0.93± 0.03) ***	(5.20 ±0.14) ****	(0.18± 0.008)
Clopidogrel (25 mg,kg, p.o) + ISO	(0.96 ±0.03) ***	(5.67± 0.04) *	(0.17 ±0.008)

The values expressed are mean ± S.E.M. (n=10). The statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnet post hoc test. P values <0.05 were considered significant.

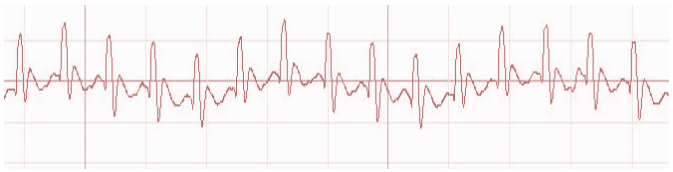
# indicates significantly different from control group at P < 0.05
*indicates significantly different from ISO group at P < 0.05
***indicates significantly different from ISO group at P < 0.001
****indicates significantly different from ISO group at P < 0.0001

Effect of Clopidogrel on ECG pattern and left ventricular function

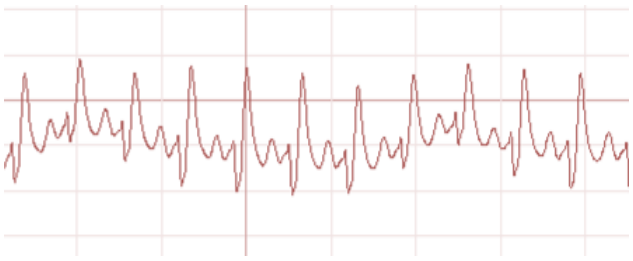
Electrocardiographic pattern of normal and experimental animals is shown in Fig 1. ECG pattern did not show any change in control rats. ISO-treated rats showed noticeable ST-segment elevation and pathological Q wave indicative of myocardial infarction. Whereas Vitamin C and Clopidogrel treated rats did not show these pathological changes in ECG. Treatment of rats with ISO (85 mg/kg, s.c.) impelled a significant increase in heart rate (HR), Mean blood pressure (BP) and left ventricular end-diastolic pressure (LVEDP) when compared to control, indicating LV haemodynamic overload. On the other hand, ISO caused significant decrease in coronary flow rate dp/dt max and dp/dtmin. compared to control. Pre-treatment with ascorbic acid and Clopidogrel improved the heart function, BP, HR, coronary flow rate, dp/ dt max, dp/dt min and LVEDP as demonstrated in Table 2. However, insignificant increase in BP and insignificant decrease in HR was observed with clopidogrel treated rats.



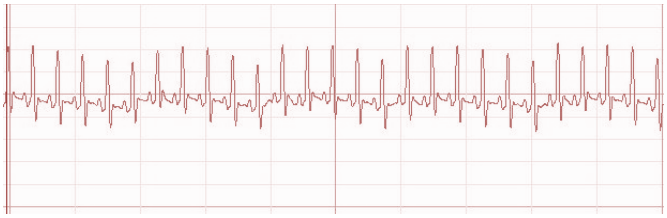
Normal



ISO (85 mg/kg)



Ascorbic acid (250 mg/kg p.o.) + ISO



Clopidogrel (25 mg/kg p.o.) + ISO

Figure 1 Effect of Clopidogrel on ECG pattern in ISO induced myocardial infarction in rats

Table 2 Effect of Clopidogrel on left ventricular function

Experimental Groups	Coronary flow rate (ml/min.)	LVEDP (mmHg)	dp/dt max. (mmHg/sec)	dp/dt min. (mmHg/sec.)	Heart rate (BPM)	Mean BP (mmHg)
Normal control	18.85± 01.10	2.49± 0.35	3408± 125.3	3115 ±47.14	164.85± 38.26	140.5± 15.45
ISO control	08.53± 01.22####	23.23 ±3.90###	1669± 60.67####	1311 ±31.90####	315.15± 41.63##	82.26± 13.07##
AA (250 mg/kg, P.O.)	17.68± 01.10****	7.05± 2.79**	2950 ±96.48****	2378± 41.64****	127.04± 11.79**	130.9± 07.68*
clopidogrel (25 mg/kg, P.O.)	12.48± 0.94*	12.22 ±0.55*	2980± 26.76****	2371 ±96.32****	312.65± 32.96	120.1± 08.45

The values expressed are mean ± S.E.M. (n=10). The statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnet post hoc test. P values <0.05 were considered significant.

#indicates significantly different from control group at P < 0.05
*indicates significantly different from ISO group at P < 0.05
**indicates significantly different from ISO group at P < 0.01
***indicates significantly different from ISO group at P < 0.001
****indicates significantly different from ISO group at P < 0.0001

Effect of Clopidogrel on biochemical parameters and antioxidant enzymes

Effect of Isoproterenol and Clopidogrel on various cardiac marker enzymes including CK-MB, LDH and Troponin-I are shown in Table 3. The activities of these enzymes were significantly increased in isoproterenol induced rats as compared to normal control rats. Clopidogrel and vit C pre-treatment in isoproterenol treated animals significantly decreased (p<0.0001for LDH, p<0.01 for Troponin-I and p<0.0001 for CKMB) the activity of cardiac marker enzymes. The effect of Clopidogrel on electrolytes levels are shown in Table 4. The level of sodium was significantly increased and potassium was significantly decreased (p<0.001) in ISO injected rats compared to the control rats. Pre-treatment with Ascorbic acid and Clopidogrel significantly prevented the rise in sodium levels while significantly decreases the level of potassium.

The levels of MDA along with the activities of the antioxidant enzymes GSH, SOD and Catalase in normal and experimental rats are listed in Table 5. Isoproterenol treated rats showed significantly increased level of MDA (p<0.05) end product of

lipid peroxidation and marker for oxidative stress in heart tissue as compared to normal control rats. ISO treatment significantly reduce ($p<0.0001$ in GSH while $p<0.05$ in Catalase) antioxidant enzyme levels. Pre-treatment with Clopidogrel and Vit C in ISO intoxicated rats insignificantly reduce the level of MDA increase the level of SOD while it significantly increases ($P<0.05$) catalase activity.

Table 3 Effect of Clopidogrel on cardiac marker enzymes

Experimental Groups	CK-MB Level (U/L)	LDH Level (U/L)	Troponin-I level (ng/ml)
Normal control	(209.35±50.76) 7.661±1.667	(4.74±0.72)	(7.661±1.667)
ISO control	(394.4±59.44##)	(1261.00±127.9####)	(22.09±7.834#)
AA (250 mg/kg, P.O.)	(96.28±19.40****)	(7.29±1.15****)	(6.52±1.966*)
clopidogrel (25 mg/kg, P.O.)	(137.48±13.47****)	(6.40±0.48****)	(6.351±1.503**)

The values expressed are mean ± S.E.M. (n=10). The statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnet post hoc test. P values <0.05 were considered significant.

#indicates significantly different from control group at P < 0.05
*indicates significantly different from ISO group at P < 0.05
**indicates significantly different from ISO group at P < 0.01
****indicates significantly different from ISO group at P < 0.0001

Table 4 Effect of Clopidogrel on serum electrolytes level

Experimental Groups	Na^+ conc.(mmol/l)	K^+ conc.(mmol/l)	Ca+2 conc. (mg/dl)
Normal control	(103.29±2.84)	(5.44±0.20)	(9.20±1.20)
ISO control	(406.28±59.53)####	(3.67±0.24) ##	(10.99±0.60)
AA (250 mg/kg, P.O.)	(112.06±2.90)****	(5.18±0.35) **	(11.96±0.62)
clopidogrel (25 mg/kg, P.O.)	(111.00±2.03)****	(7.56±0.55)****	(9.98±0.64)

The values expressed are mean ± S.E.M. (n=10). The statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnet post hoc test. P values <0.05 were considered significant.

#indicates significantly different from control group at P < 0.05
**indicates significantly different from ISO group at P < 0.01
**** indicates significantly different from ISO group at P < 0.0001

Table 5 Effect of Clopidogrel on various anti-oxidant and oxidative parameters

Experimental Groups	GSH (µg/ml)	SOD (µg/ml)	MDA (µg/ml)	Catalase (mmol/g of tissue)
Normal control	(76.51±15.97)	(85.90±30.27)	(0.07±0.003)	(20.27±3.89)
ISO control	(4.70±1.03) ####	(43.79±8.61)	(0.23±0.04) #	(6.29±1.50) #
AA (250 mg/kg, P.O.)	(10.00±0.34)	(266.8±158.2)	(0.10±0.01)	(13.91±2.03)
clopidogrel (25 mg/kg, P.O.)	(3.05±0.18)	(62.66±18.71)	(0.08±0.01)	(15.30±0.42)*

The values expressed are mean ± S.E.M. (n=10). The statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnet post hoc test. P values <0.05 were considered significant.

#indicates significantly different from control group at P < 0.05
*indicates significantly different from ISO group at P < 0.05

Effect of Clopidogrel on Caspase-3 enzyme level

Isoproterenol administration showed a significant increase ($P<0.01$) in caspase-3 level as compared to normal control rats while pretreatment with Clopidogrel in ISO intoxicated rats showed significant decrease ($p<0.01$) and Ascorbic acid also decreases ($p<0.01$) in Caspase-3 levels shown in Figure 4.

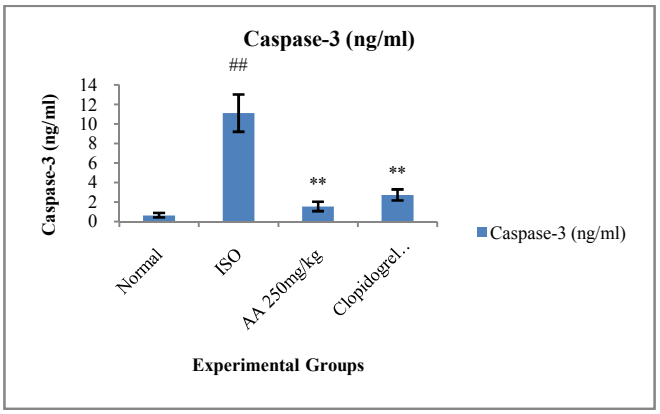


Figure 2 Effect of Clopidogrel on Caspase-3 in ISO induced myocardial infarction in rats

The values expressed are mean ± S.E.M. (n=10). The statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by dunnett's post hoc test. P values <0.05 were considered significant.

indicate significant difference from normal control rats.
*indicate significant difference from ISO control rats.
##indicates significantly different from normal control group at P < 0.01
**indicates significantly different from ISO group at P < 0.01

Effect of Clopidogrel on IL-6 and TNF-α gene expression study

Isoproterenol administration showed a significant upregulation in inflammatory markers such as IL-6 and insignificant upregulation in *TNF-α* mRNA expression as compared to normal control rats while pretreatment with Clopidogrel in ISO intoxicated rats showed significant down regulation in IL-6 ($p<0.0001$) and insignificant downregulation in *TNF-α* mRNA gene expression as shown in Figure 3 and 4.

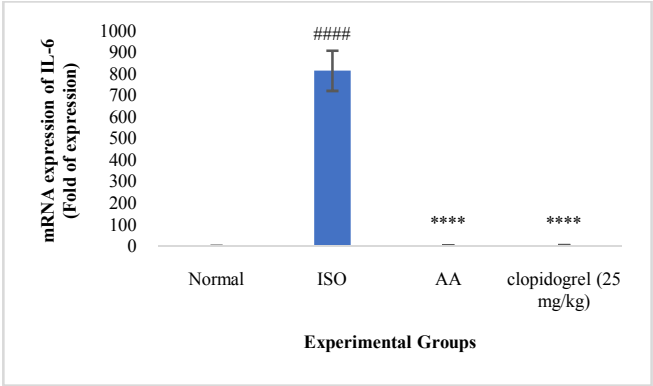


Figure 3 Effect of Clopidogrel on IL-6 in ISO induced myocardial infarction in rats

The values expressed are mean ± S.E.M. (n=10). The statistical analysis was carried out by one-way analysis of variance

(ANOVA) followed by dunnett's post hoc test. P values <0.05 were considered significant.

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####indicates significantly different from normal control group at P < 0.0001
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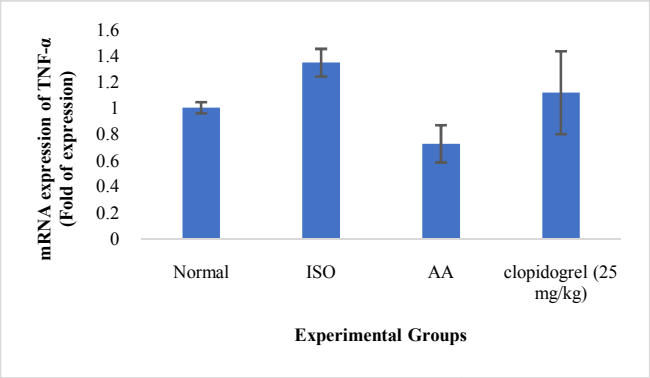

Figure 4 Effect of Clopidogrel on TNF-α gene expression study in ISO induced myocardial infarction in rats

The values expressed are mean ± S.E.M. (n=10). The statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by dunnett's post hoc test. P values <0.05 were considered significant.

Effect of Clopidogrel in TTC staining

Figure 5 shows the heart sections of normal and ISO injected rats stained with TTC. ISO injected rats showed increased infracted area shown by yellowish colour compared to the control rats. Treatment with Clopidogrel and Vitamin C shows for 14 days then intoxicated with ISO shows decrease in infarct staining as compared to ISO treated rats.

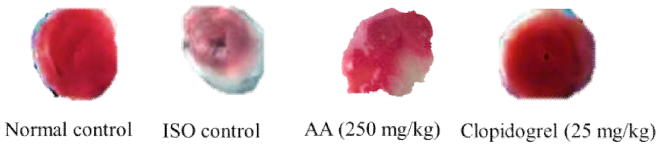

Figure 5 Representative triphenyl tetrazolium chloride stained heart slices from Normal control, ISO control, AA (250 mg/kg) and Clopidogrel (25 mg/kg). The white area presented infarcted area

Effect of Clopidogrel in Histopathology

Histopathological examination showed normal cardiac fibres and no inflammatory cell and infarction in the myocardium of control animals. Heart tissues of ISO group revealed myonecrosis, myofibers loss and increased number of inflammatory cells as compared to control group. Pre-treatment with Clopidogrel resulted in a decrease in the degree of inflammatory cells and the morphology of cardiac muscle fibers was relatively well preserved with no evidence of focal necrosis. (Fig. 6)

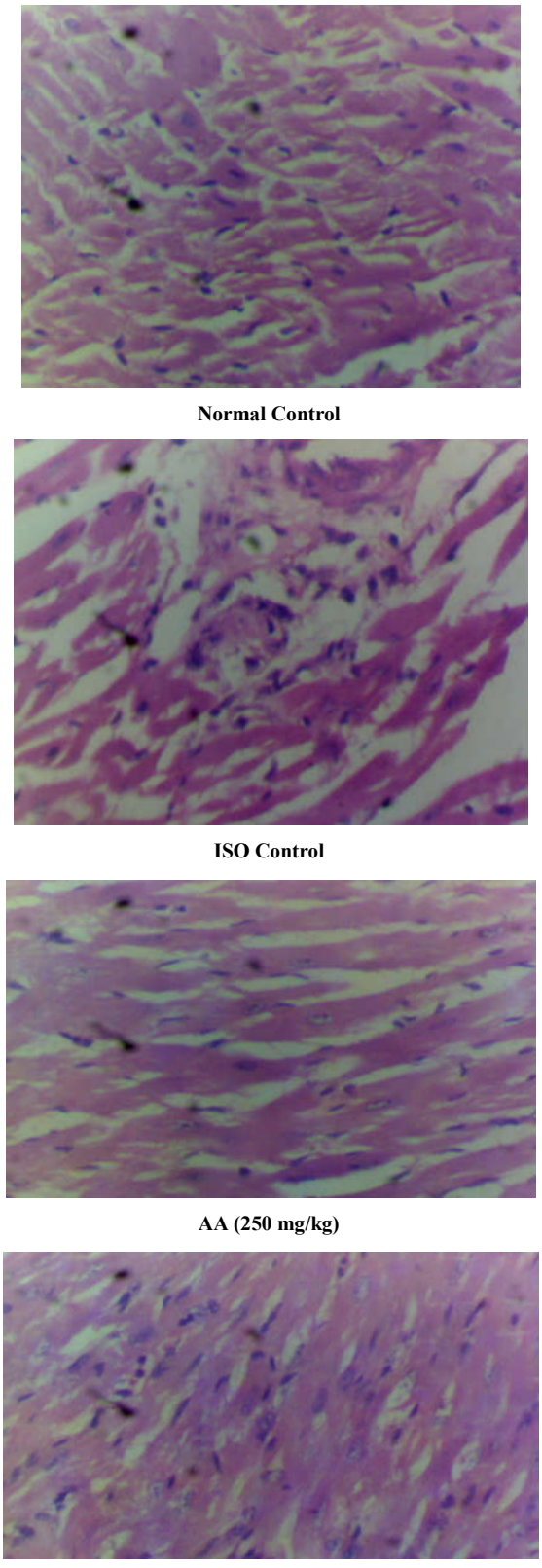

Figure 6 Effect of prasugrel on Histopathology in ISO induced myocardial infarction in rats

DISCUSSION

Platelets play a pivotal role in promoting systemic and cardiac inflammatory responses in post-MI. Platelets accumulate within the infracted myocardium, contributing to regional

inflammation, ventricular remodelling and rupture of myofibers. Antiplatelet therapy reduces the severity of inflammation and risk of post-MI complication, demonstrating a previously unrecognized protective action (Liu *et al.*, 2011).
MI induced by injection of ISO is a standardized model to study the beneficial effects of numerous drugs and antioxidants. In the present study, we found that Clopidogrel treatment exerts a strong cardioprotection in ISO induced MI in rats.

Following isoproterenol administration, the heart weight increased significantly, with relatively significant changed tibial length resulting in the increase of the heart weight to tibia length ratio. Increase in heart weight might be attributed to increased water content, edematous intramuscular space (Upaganlawar *et al.*, 2009) and increased protein content. These results are in consistent with the previous report (Judd and Wexler, 1974), which has observed extensive edematous intramuscular space, accumulation of mucopolysaccharides and cellular infiltration after 4 h of induction of myocardial infarction. It has been proposed that a 1% increase in myocardial water content could be expected to result in possibly a 10% reduction in myocardial function (Laine and Allen, 1991). Pre-treatment of Clopidogrel brings down the heart weight to tibial length ratio indicative of its protection of myocardium against infiltration and it also could be due to the decrease in water content of the myocardium.

Significant changes in ECG patterns were observed in ISO-induced rats when compared with control rats. Study results noticed the appearance of pathological Q waves and ST segment elevation, which are characteristic signs of infarction. The consecutive loss of cell membrane in injured myocardium results in electrocardiograph abnormalities like ST-segment elevation (Gong *et al.*, 2012). Clopidogrel pre-treatment markedly inhibited ISO-induced ST-segment elevation which indicates its cell membrane preserving action.

Left ventricular (LV) dysfunction after myocardial infarction is major predictors of death (Wang *et al.*, 2011). In our finding, prominent left ventricular dysfunction was observed in ISO-treated rats and improvement of LV function was observed in Clopidogrel pre-treated groups when compared to ISO group animals.

Myocardium contains plentiful concentrations of diagnostic markers of myocardial infarction and once metabolically damaged, it releases its contents into the extracellular fluid (Upaganlawar *et al.*, 2009). Of all the macromolecules that leak from the damaged tissue, enzymes because of their tissue specificity and catalytic activity are the best markers of tissue damage. Assay of the activity of CK-MB and cTnI in serum is some important diagnostic parameters, because of the marked abundance of this enzyme in myocardial tissue and virtual absence from most of other tissues and its consequent sensitivity. cTnI and CK-MB isoenzyme activity is useful not only as an index of early diagnosis of myocardial infarction, but also any type of myocardial injury. The amount of these cellular enzymes in serum reflects the alterations in plasma membrane integrity and/or permeability (Sabeena Farvin *et al.*, 2004).

In the present study isoproterenol injected rats showed significant elevation in the levels of these marker enzymes in serum, which were in line with the previous reports and indication of isoproterenol induced necrotic damage of the myocardium and leakiness of the plasma membrane. Clopidogrel pre-treatment resulted in the dropped the activity of these marker enzymes in serum which demonstrated that Clopidogrel could maintain membrane integrity thereby restricting the leakage of these enzymes.

In the cell, ATPases are closely associated with the plasma membrane and participate in the energy dependent transport of sodium, potassium, magnesium and calcium translocation. An increase in sodium and calcium along with decrease in potassium were observed in ISO injected rats. Increased concentration of sodium might be due to decrease in Na + /K+ ATPase (S Al-Numair *et al.*, 2011). Depletion of ATP by ISO leads to opening of K+ channel leading to the decrease in K+ ions in the myocardial tissue. Increased levels of intracellular Na+ also operate to depress Ca2+ effect and augment Ca2+ influx. The reversal in ISO-induced alteration in ion concentration like Na^{+}, Ca^{+2}, and K^{+} was observed on pre-treatment with ascorbic acid and Clopidogrel when compared to ISO group.

ISO produces myocardial necrosis which causes cardiac dysfunction, increased lipid peroxidation, altered activities of the cardiac enzymes and antioxidants (Patel *et al.*, 2010). Many pieces of evidence demonstrate the cardiotoxicity due to isoproterenol like the generation of cytotoxic free radicals through auto-oxidation of catecholamines, increased cyclic adenosine monophosphate, increased intracellular Ca^{2+} overload, depletion of high-energy phosphate stores and oxidative stress (Garg and Khanna, 2014).

ISO administration has been reported to induce severe oxidative stress and lipid peroxidation process (Rathore *et al.*, 1998). Overproduction of reactive oxygen species can cause severe impairment of cellular functions and necrotic lesions in the myocardium of rats. In the other hand, superoxide dismutase, catalase, and glutathione peroxidase constitute a mutually supportive enzyme team of defence against oxidative injury (Ji *et al.*, 1988). In our study, we found an increase in the level of a stable degradation product of the oxygen-derived free radicals and lipid peroxides, MDA. Besides MDA, decrease in levels of GSH, SOD and CAT further confirmed the occurrence of oxidative stress in ISO group. Clopidogrel pre-treatment reverses the changes of oxidative and anti-oxidative enzyme levels.

The present study exhibited a significant increase in caspase-3 activity in ISO injected rats. Increased caspase-3 protease activity indicates cardiac apoptosis in the present study. Study have reported that β-AR stimulation by catecholamine induces cardiac apoptosis or/and necrosis (Remondino *et al.*, 2003). Clopidogrel treatment presented significant reduction in caspase-3 protease activity. This indicates that Clopidogrel prevents apoptosis in ISO injected MI in rats.

Inflammatory cytokine markers such as IL-6 and *TNF-α* are upregulated in myocardial injury states, ischemia–reperfusion, chronic ischemia post-myocardial infarction, and heart failure (Ramani *et al.*, 2004). In the present study isoproterenol

administration showed insignificant upregulation in mRNA of *TNF-α* and significant upregulation in IL-6 levels indicative of necrosis induced inflammation of myocardial tissue. In Clopidogrel pre-treated group, significantly downregulation in levels of inflammatory markers indicated that the Clopidogrel pre-treatment suppressed the release of inflammatory markers viz. IL-6 and *TNF-α*.

Previous studies have shown an increase in infarct area due to isoproterenol as indicated with white to yellow colour by using TTC staining (Devika and Mainzen Prince, 2008). It has been reported that the area of infarction shows loss of membrane integrity that occurs because of the release of lactate dehydrogenase (Devika and Mainzen Prince, 2008; Nivethetha *et al.*, 2009). Pre-treatment with Clopidogrel and Ascorbic acid significantly decreased the myocardial infarct area by TTC staining.

Histopathological examination of myocardial tissue in control illustrated clear integrity of the myocardial cell membrane and no inflammatory cell infiltration was observed. Isoproterenol injected rats showed coagulative necrosis, separation of cardiac muscle fibers and infiltration of inflammatory cells. The reduced inflammatory cell infiltration and normal cardiac muscle fiber architecture further confirmed the cardioprotective effect of Clopidogrel.

In conclusion, the present study showed that Clopidogrel unveiled cardioprotection by restoring most of the altered physical, biochemical, haemodynamic parameters, maintaining antioxidant status, preventing cellular damage, anti-apoptotic markers and anti-inflammatory markers by suppression of caspase-3 level and downregulating the mRNA expression of IL-6 and *TNF-α*.

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