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Sodium current inhibitor ranolazine ameliorates experimentally induced diabetic cardiomyopathy

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ABSTRACT: Ranolazine is an anti-ischemic drug with glucose lowering effect. Our study scrutinized the effect of ranolazine on Streptozotocin (STZ) induced diabetic cardiomyopathy, emphasizing role of Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA) pump. STZ induced diabetic rats showed significant hyperglycaemia with weight loss, hyperlipidaemia, increased cardiovascular risk indices as well as atherogenic index of plasma, Left Ventricular (LV) dysfunction, abnormal electrocardiography (ECG) and elevated cardiac biomarkers (CK-MB, LDH and AST). Twelve weeks ranolazine treatment ameliorated diabetes associated biochemical alterations and LV function along with ECG. The diabetic heart showed increased lipid peroxidation and compromised antioxidant defence mechanism which was reversed by ranolazine treatment. Reduced SERCA expressions were recognised in STZ treated diabetic rats. Ranolazine amplified SERCA expressions thus by regulating intracellular calcium homeostasis and keeping diabetic cardiomyopathy at bay. Ranolazine also prevented histological alterations in the heart and pancreas. Our results may open novel avenues for designing treatment strategies using ranolazine against diabetic cardiomyopathy.

KEYWORDS: Diabetic cardiomyopathy; streptozotocin; ranolazine; sarcoplasmic endoplasmic reticulum calcium ATPase

1. INTRODUCTION

Diabetic cardiomyopathy (DCM), which is generally recognized as diabetes associated abnormalities in myocardial structure and function, without coexistence of other cardiac disorders *viz*. coronary atherosclerosis, valvular heart disease, hypertension and congenital heart disease [1]. The cardinal characteristics of DCM include fibrosis, hypertrophy, apoptosis, diastolic and systolic dysfunction, which may lead to heart failure. The occurrence of DCM is escalating in parallel with increase in diabetes mellitus [2]. The diabetic patients have increased risk of developing heart failure up to 74%, and are four times more likely to die than those without heart failure (HF) [3]. Literature suggests that in type 1 diabetes mellitus, 1% rise in HbA1c was associated with 30% increase in risk of HF whereas in type 2 diabetes mellitus, 8% risk of DCM is increased [2]. Accumulating evidence from preclinical and clinical studies suggests multifactorial pathogenesis of DCM [4, 5]. The classical mechanisms includes impairment of intracellular calcium homeostasis which contribute to impaired cardiac function and autonomic neuropathy; oxidative stress which damages the heart causing cardiomyocyte death; abnormal cardiomyocyte energy metabolism provoking lipotoxicity; hyperglycaemia and accumulation of advanced glycation end-products (AGEs) responsible for cardiac fibrosis followed by diastolic dysfunction leading to heart failure [4, 5]. At molecular level, peroxisome proliferator-activated receptors, protein kinase C, O-linked N-acetylglucosamine, DNA methylation, histone acetylation, and dysregulation of microRNAs have been implicated in development of DCM [2, 5].

At present, various classes of oral hypoglycaemic agents, such as biguanides, sulfonylureas, meglitinides, thiazolidinediones, α-glucosidase inhibitors, DPP-4 inhibitors, GLP-1 receptor agonists and SGLT2 inhibitors are available [1, 3]. Even with intensive glycaemic control, underlying pathological alterations that promote DCM is not reversed. Thus, targeting pathological network driving the persistence of cardiac dysfunction in spite of tight glycaemic control can be proposed as a novel strategy to ameliorate DCM.

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Ranolazine is an FDA approved anti-anginal drug. Various preclinical [6-8] and clinical studies [9, 10] demonstrated ranolazine exert antidiabetic actions by increasing insulin release, β cell survival and lowering serum glucose levels [6-8]. Role of ranolazine has been established in numerous cardiac disorders like ventricular arrhythmias, atrial fibrillation, myocardial infarction, diastolic dysfunction, systolic heart failure, and chemotherapy-induced cardiotoxicity [11]. At therapeutic concentrations, ranolazine prevented the hypertrophic cardiomyopathy in transgenic mice carrying troponin T mutations [11, 12]. The cardioprotective effect of ranolazine is believed to be due to its inhibitory action on late sodium current, thus by maintaining sodium-calcium homeostasis [13]. Consistent with this finding, ranolazine has shown remarkable effect in reducing calcium overload by limiting accumulation of it in isolated heart [14]. Also, *in-vivo* study has proven that ranolazine reverses progressive LV dysfunction by stabilizing SERCA [15]. Furthermore, it is known to inhibit cardiac fatty acid oxidation which decreases oxidative stress improving cardiac function [16]. Ranolazine also showed antioxidant properties *in-vitro*. [17] Of note, above mentioned studies throw light on potential of ranolazine in handling cardiac disorders. However, the protective effect of ranolazine against DCM has not yet been demonstrated. Therfore, a search for cardioprotective effect of Ranolazine which can interfere the early manifestations and possibly halt later devlopment of diabetic cardiomyopathy was targeted in present study. The study comprises the effect of ranolazine on early and late pathological markers of diabetic cardiomyopathy.

2. RESULTS

2.1. Effect on growth rate in STZ induced DCM

In STZ induced diabetic rats, the significant reduction in the body weight is reported in various studies [18, 19]. In present study, when body weight was calculated in terms of growth rate, the significant (P<0.01) decrease was found from day 7 to day 21 as compared to normal control animals. Ranolazine group growth curve resembles normal control growth curve, suggesting ranolazine did not affect development of animals (Figure 1).

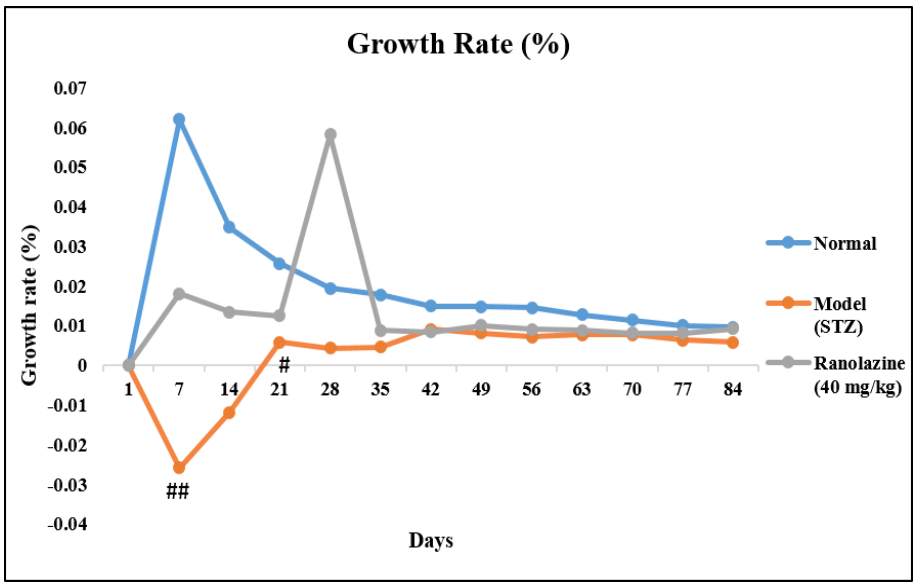

Figure 1. Effect of ranolazine on growth rate in STZ induced DCM.

Values are expressed as Mean ± SEM. Values are statistically evaluated using two-way ANOVA analysis followed by Bonferroni's Post hoc test. Significant values were compared with #P<0.05 normal control vs. model control ##P<0.01 normal control vs. model control.

2.2. Effect on glucose in STZ induced DCM

Hyperglycaemia was successfully induced by injecting STZ in rats. The glucose levels in model control animals were found to be significantly (P<0.001) elevated than normal control animals. Treatment with ranolazine significantly (P<0.0001) decreased glucose levels as compared to model control animals confirming its anti-diabetic effect (Figure 2).

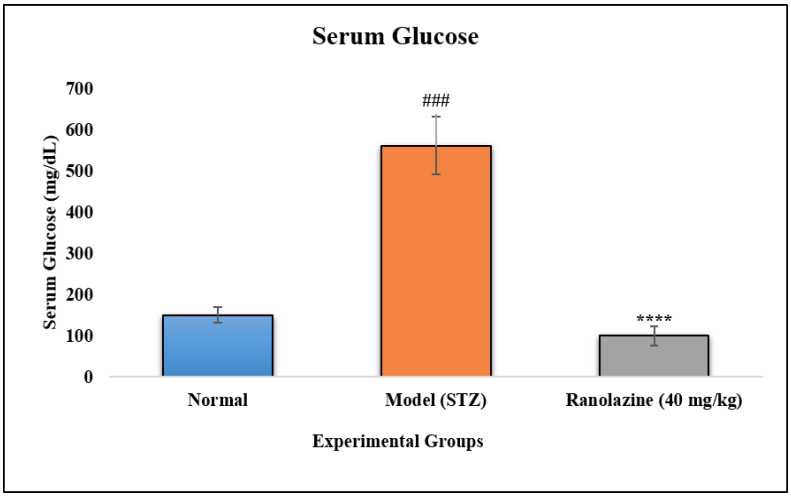

Figure 2. Effect of ranolazine on serum glucose in STZ induced DCM.

Values are expressed as Mean ± SEM. Values are statistically evaluated using one-way ANOVA analysis followed by Dunnett's Post hoc test. Significant values were compared with ###P<0.001 normal control vs. model control; ****P<0.0001 model control vs. Ranolazine 40mg/kg

2.3. Effect on lipid profile in STZ induced DCM

In diabetic state, due to decrease in glucose uptake by cardiac cells, the fatty acid is used as a fuel [20]. The oxidation of this causes increase in lipid levels which are also observed in the current study. The significant increase in levels of triglyceride (P<0.0001), total cholesterol (P<0.0001), LDL (P<0.05), and VLDL (P<0.0001) were observed in model control animals as compared to normal control animals. With ranolazine treatment of 12 weeks, the significant decrease in levels of triglyceride (P<0.0001), total cholesterol (P<0.0001), LDL (P<0.05), and VLDL (P<0.0001) were observed as compared to model control animals. HDL was significantly (P<0.001) decreased in model control animals as compared to normal control animals. Ranolazine treatment combat this decrease in HDL levels significantly (P<0.01), thus by improving lipid profile (Figure 3). The cardiovascular risk indices and atherogenic index of plasma were determined to assess the impact of hyperlipidemia on the heart and blood vessels. The pronounced and significant increase in cardiac risk indices (P<0.0001) and AIP (P<0.001) were observed in STZ induced diabetic rats as compared to normal control animals. The significant protective effect of ranolazine was observed on risk indices (P<0.0001) and AIP (P<0.001) evident by their decrease as compared to model control (Table 1).

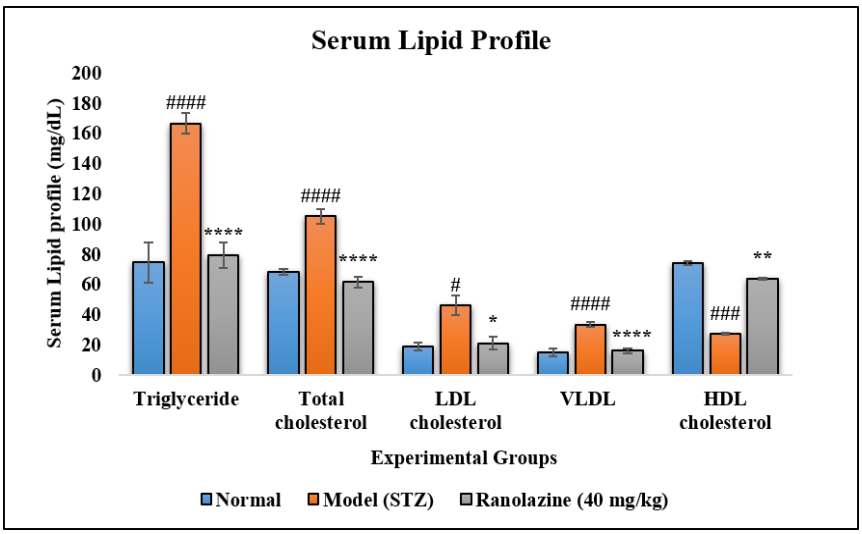

Figure 3. Effect of ranolazine on serum lipid profile in STZ induced DCM.

LDL Cholesterol, Low-density lipoprotein cholesterol; VLDL, Very low-density lipoprotein; HDL Cholesterol, High-density lipoprotein. Values are expressed as Mean ± SEM. Values are statistically evaluated using one-way ANOVA analysis followed by Dunnett's Post hoc test. Significant values were compared with #P<0.05, ###P<0.001, and ####P<0.0001 normal control vs. model control; *P<0.05, **P<0.01, and ****P<0.0001 model control vs. Ranolazine 40mg/kg.

Table 1. Effect of ranolazine on cardiovascular risk indices, AIP and hemodynamic parameters in STZ induced DCM.

Experimental Groups	Cardiac risk indices		Atherogenic index of plasma (AIP)	LVEDP (mmHg)	+ dP/dt $_{max}$ (mmHg/s)	- dP/dt $_{min}$ (mmHg/s)
	Cardiac risk index 1	Cardiac risk index 2				
Normal	0.899 ± 0.013	0.253 ± 0.033	0.127 ± 0.066	8.263 ± 2.288	59.61 ± 3.717	34.74 ± 0.860
Model (STZ)	3.890 ± 0.200 (####)	1.681 ± 0.219 (###)	0.780 ± 0.020 (###)	22.99 ± 2.735 (##)	24.43 ± 0.210 (####)	18.44 ± 0.249 (##)
Ranolazine (40 mg/kg)	0.930 ± 0.061 (****)	0.329 ± 0.063 (***)	0.135 ± 0.046 (***)	6.491 ± 1.469 (**)	51.35 ± 1.207 (***)	35.05 ± 3.137 (**)

LVEDP, Left ventricular end-diastolic pressure. Values are expressed as Mean ± SEM. Values are statistically evaluated using one-way ANOVA analysis followed by Dunnett's Post hoc test. Significant values were compared with ##P<0.01, ###P<0.001, and ####P<0.0001 normal control vs. model control; **P<0.01, ***P<0.001, and ****P<0.0001 model control vs. Ranolazine 40mg/kg.

2.4. Effect on ECG in STZ induced DCM

The abnormalities in ECG can be an alarm for asymptomatic patients regarding risk of heart diseases [21]. ECG recording of non-diabetic rats was found to be normal. ST elevation and QT interval prolongation was observed in diabetic rats after 12 weeks of study period. Treatment with ranolazine preserves proper functioning of heart and reverses the functional abnormalities seen in diabetic group. ECG recording of ranolazine group was comparable to normal group (Figure 4 A-C).

2.5. Effect on LV function in STZ induced DCM

LV dysfunction was evaluated by measuring LVEDP, dP/dt_{max} and dP/dt_{min}. The significant (P<0.01) increase in LVEDP and significant reduction in dP/dt_{max} (P<0.0001) as well as dP/dt_{min} (P<0.01) after 12 weeks of STZ injection in model control animals suggested detrimental effect of STZ on heart. Rats treated with ranolazine were significantly protected from LV diastolic and systolic dysfunction as evident by improved LVEDP (P<0.01), dP/dt_{max} (P<0.001) and dP/dt_{min} (P<0.01) (Table 1).

2.6. Effect on cardiac biomarkers in STZ induced DCM

The elevation of cardiac enzymes are important indicators of myocardial damage. In current study, the STZ induced diabetic rats showed significant increase in CK-MB (P<0.001), LDH (P<0.001) and AST (P<0.0001) activity compared with normal control rats. Administration of ranolazine significantly decreased CK-MB (P<0.001), LDH (P<0.0001) and AST (P<0.001) activity in diabetic animals (Table 2).

2.7. Effect on oxidative stress in STZ induced DCM

The production of reactive oxygen is found to be increased in diabetes which increases lipid peroxidation and oxidative stress leading to diabetic cardiomyopathy [4, 22]. In present study, STZ induced diabetic rats showed significant (P<0.0001) increase in MDA levels when compared to normal control animals which was significantly (P<0.0001) prevented in ranolazine treated group, proving its role against lipid peroxidation. The enzymes *viz*. SOD, GSH and Catalase, effective to combat oxidative stress are also significantly (P<0.01, P<0.01 and P<0.0001 respectively) decreased in diabetic rats as compared to non-diabetic animals. As compared to model control, ranolazine treated animals significantly increased SOD (P<0.01) and Catalase (P<0.0001) levels showing its ROS scavenging potential. No significant difference was found in GSH levels in ranolazine treated group as compared to model control (Table 2).

2.8. Effect on SERCA expression in STZ induced DCM

The calcium overload in cardiac cells causes cardiomyopathy [23]. One of the mechanisms to overcome this is to increase level of SERCA [24, 25]. With respect to normal control group, diabetic animals showed significant decrease in SERCA levels suggesting harmful effects on heart. The beneficial effect of ranolazine on enhancing calcium reuptake was evident by significant increase in SERCA expression as compared to model control animals (Figure 5).

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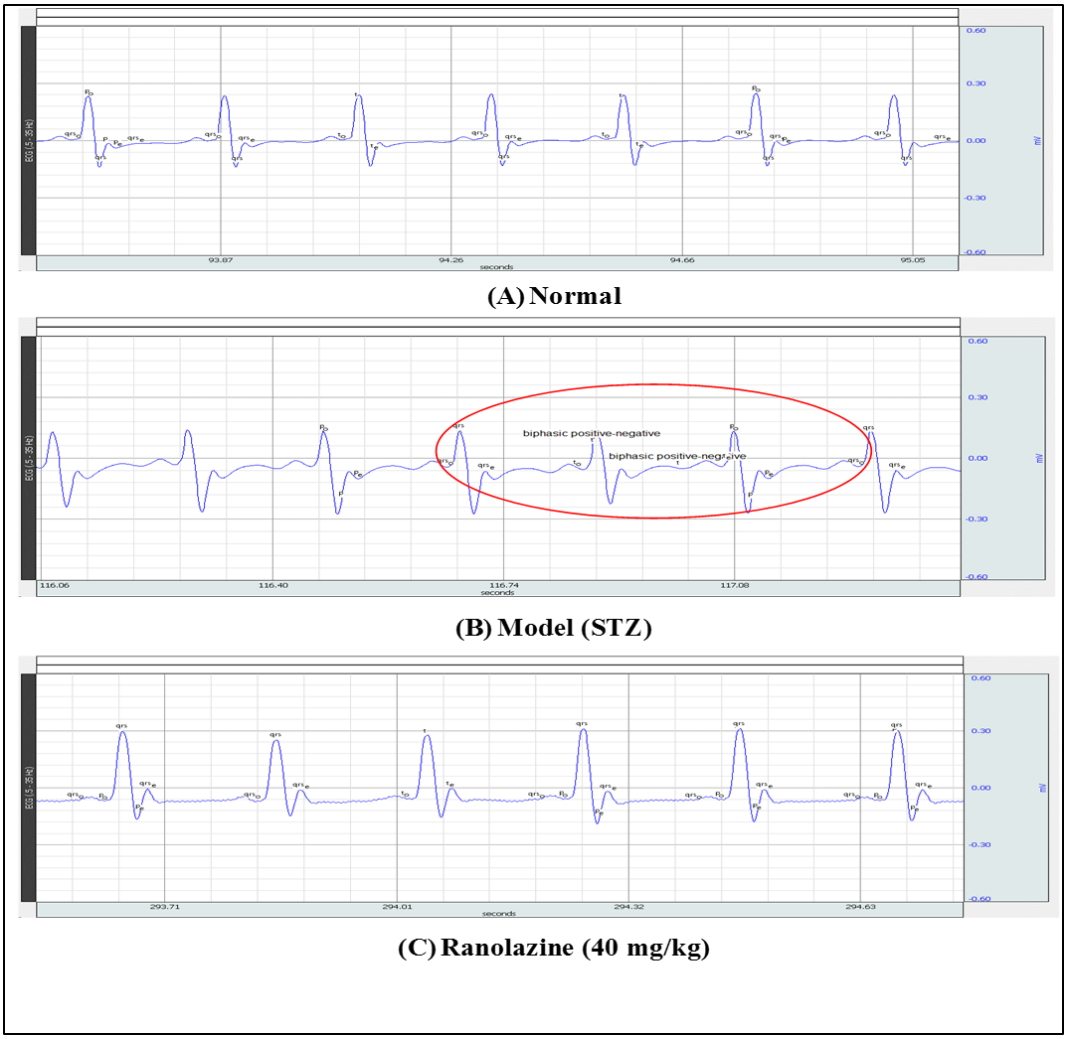

Figure 4. Effect of ranolazine on electrocardiography in STZ induced DCM.

(A) Normal: Non-diabetic rats showing normal pattern of ECG; **(B)** Model: STZ treated rats showing prolongation of QT and ST elevation **(C)** Ranolazine (40mg/kg): Treatment with ranolazine corrected the STZ induced changes in ECG pattern to normal.

Table 2. Effect of ranolazine on cardiac biomarkers and oxidative stress parameters in STZ induced DCM.

Experimental Groups	Creatine kinase (CK-MB) (U/L)	Lactate dehydrogenase (LDH) (U/L)	Aspartate aminotransferase (AST) (IU/L)	Malondial dehyde (MDA) (µg/mg of protein)	Superoxide dismutase (SOD) (unit/min/g tissue)	Glutathione (GSH) (µg/mg of protein)	Catalase (µmol of H_2O_2 /min/g tissue)
Normal	766.6 ± 77.26	675.8 ± 50.680	112.2 ± 4.265	0.014 ± 0.002	178.3 ± 29.520	2.098 ± 0.535	92.950 ± 0.475
Model (STZ)	1826 ± 220.8 (###)	1563 ± 139.7 (###)	233.9 ± 1.367 (####)	0.092 ± 0.011 (####)	49.240 ± 1.991 (##)	0.143 ± 0.041 (##)	75.700 ± 1.213 (####)
Ranolazine (40 mg/kg)	793 ± 69.83 (***)	369.3 ± 36.90 (****)	168.9 ± 11.19 (***)	0.018 ± 0.002 (****)	166.2 ± 3.611 (**)	1.264 ± 0.064	90.57 ± 0.591 (****)

Values are expressed as Mean ± SEM. Values are statistically evaluated using one-way ANOVA analysis followed by Dunnett's Post hoc test. Significant values were compared with ##P<0.01, ###P<0.001, and ####P<0.0001 normal control vs. model control; **P<0.01, ***P<0.001, and ****P<0.0001 model control vs. Ranolazine 40mg/kg.

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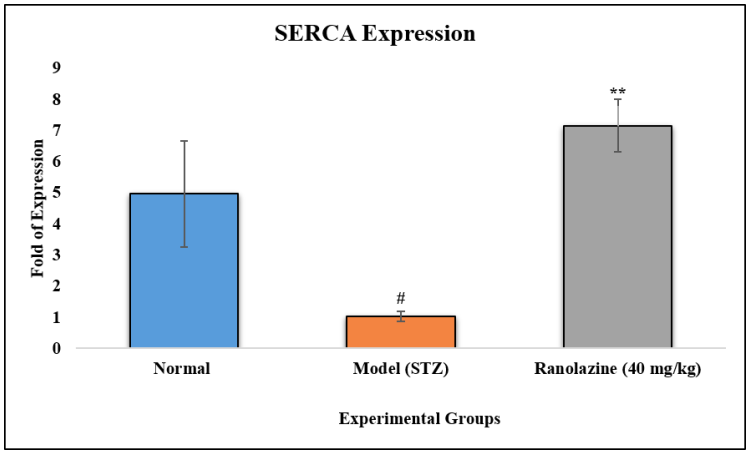

Figure 5. Effect of ranolazine on SERCA expression in STZ induced DCM.

Values are expressed as Mean ± SEM. Values are statistically evaluated using one-way ANOVA analysis followed by Dunnett's Post hoc test. Significant values were compared with #P<0.05 normal control vs. model control; **P<0.01 model control vs. Ranolazine 40mg/kg.

2.9. Effect on Histopathology in STZ induced DCM

The histopathological analysis of non-diabetic animals showed normal architecture of heart with definite shape of nuclei. In model control rats, disorganization, and degeneration of myofibers and vacuole formation were observed as compared to normal control animals. Swelling of myofibers and interstitial hemorrhage were also noticed in model control rats. Compared with the diabetic cardiomyopathy group, all these changes were reversed by ranolazine treatment except swelling of myofibers (Figure 6 A-C).

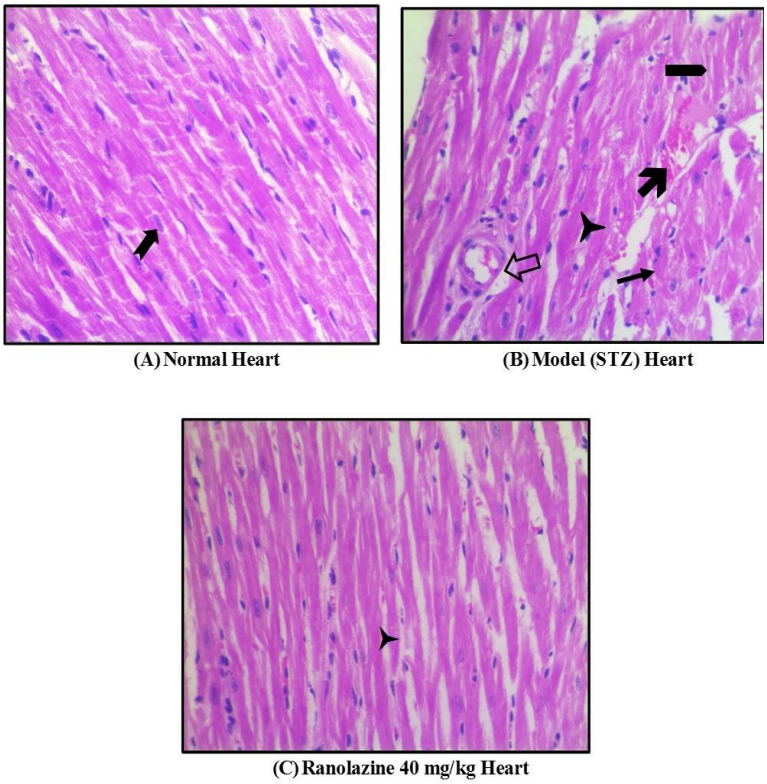

Figure 6. Effect of ranolazine on heart histopathology in STZ induced DCM.

(A) Normal: Shows normal histology of heart with intact structure and nuclei (➡) ; **(B) Model (STZ)**: Diabetic control rats showing disorganization (▬), degeneration (➡), and swelling of myofibres (➤), interstitial hemorrhage (➔) and vacuole formation (⇨); **(C) Ranolazine (40mg/kg)**: Improved morphology can be observed with less myocardial fibre swelling (➤). (Haematoxylin and Eosin, magnification 40X).

The normal architecture of endocrine and exocrine pancreas were observed in H&E stained sections of pancreas of normal control animals. STZ treated animals showed focal decreased in islet size and disrupted boundary of islet of Langerhans. Necrotic changes were also observed along with disrupted pancreatic acinar cells. Ranolazine treatment preserves the microscopical structure of pancreas (Figure 7 A-C).

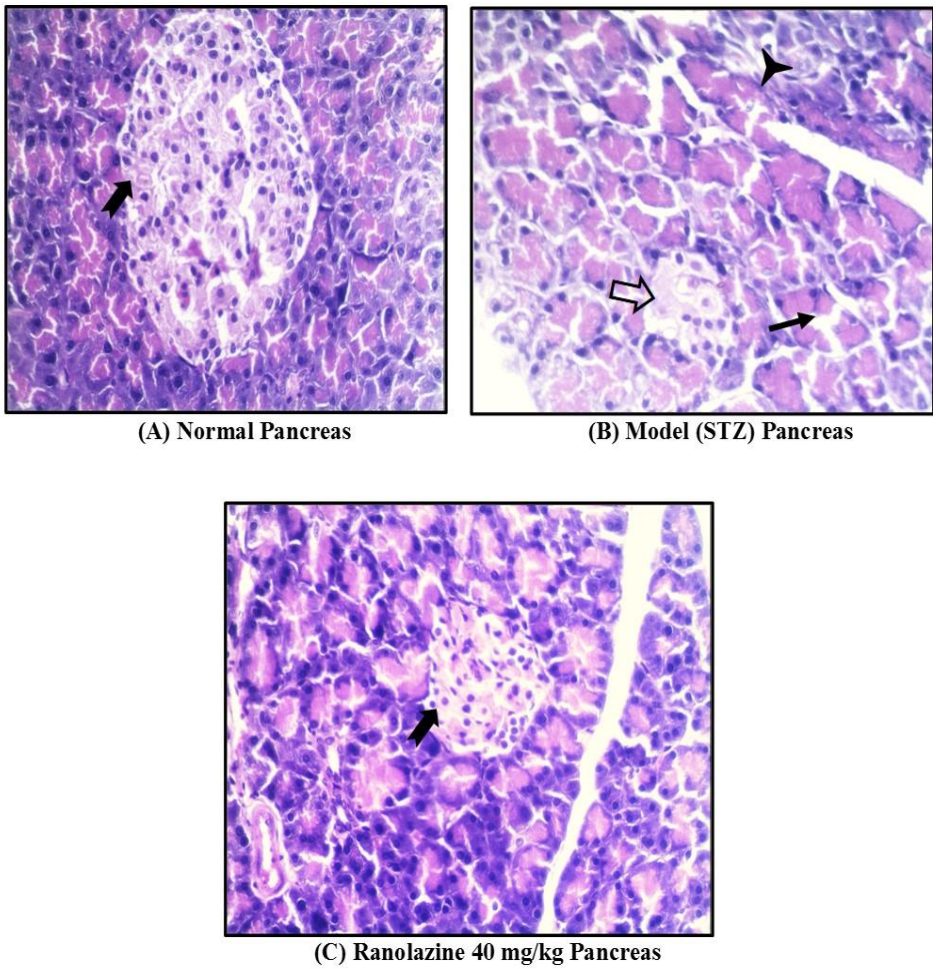

Figure 7. Effect of ranolazine on pancreas histopathology in STZ induced DCM.

(A) Normal: Pancreas of normal control rats showing normal architecture of pancreatic cells (➡) ; **(B) Model (STZ)**: Diabetic control rats showing decrease in islet size with disrupted boundary of islet of Langerhans (⇨), necrotic changes (➢) and disrupted acinar cells (➞); **(C) Ranolazine (40mg/kg)**: Ranolazine treated rats preserves normal structure of pancreas (➡). (Haematoxylin and Eosin, magnification 40X).

3. DISCUSSION

The major novel result of present 12 weeks study is that ranolazine exerts a pronounced cardio-protective effect in STZ-induced diabetic cardiomyopathy. The measurement of blood glucose level is the most common means of weighing metabolic control in diabetes. In the current study, STZ treated animals showed increase in blood glucose levels with decrease in body weight. The treatment with ranolazine resulted in significant improvement in glycaemic control which is in consistent with similar published preclinical [6-8] and clinical studies [9, 10]. The preservation as well as decreased apoptosis of β- cell and glucose stimulated insulin secretion can be attributed to glucose lowering effect of ranolazine. Also, it is reported as partial fatty acid oxidation inhibitor which halts gluconeogenesis in condition of insulin deficiency [16]. The effect might also be due to normalisation of body weight in ranolazine treated groups as compared to model control [8, 11].

In STZ-induced diabetes, along with elevated blood glucose there is rise in lipid levels which are associated with deterioration of contractile function and increased myocardial injury indexes in blood. Insulin deficiency decreases glucose delivery in cardiomyocytes and use of fatty acids as an alternative energy source.

This elevation increases subsequent triglyceride synthesis in the heart leading to cellular lipotoxicity and the beginning of cardiac dysfunction [20]. In accordance with these reports, in the present study, hyperlipidemia characterized by increased levels of cholesterol, triglycerides, LDL and VLDL were observed in untreated diabetic animals. Along with this, marked decrease in HDL was also observed. Interestingly, all the lipid abnormalities developed by STZ were counteracted by ranolazine treatment. This effect might be partly due to control on glucose levels.

STZ induced diabetic animals produced significant changes in hemodynamic parameters [26]. The dP/dt_{max} and dP/dt_{min} are the maximum rates of pressure development during contraction and relaxation whereas LV end-diastolic pressure (LVEDP) reflected LV relaxation. As reported in various studies, in our study also diabetic animals showed reduced dP/dt_{max} and elevated LVEDP suggesting diastolic dysfunction and reduced dP/dt_{min} suggesting systolic dysfunction. Ranolazine treated group provides sufficient contractile reserve to improve detrimental effect of diabetes on cardiac contractility. Also, in diabetic rats, the electrocardiogram showed prolongation of QT interval, which is essential manifestation of diabetic neuropathy and is been used to screen risk for sudden cardiac death in diabetic patients. The normal ECG was observed with ranolazine treatment which might be due to improvement in glycaemic control and lipid profile. Also, reduced myocardial remodelling might be contributed to possible long-term effects of late sodium current (I_{Na}) inhibition by ranolazine [11].

Hyperglycaemia causes serious insult to the heart muscle and evoking release of enzymes (like CK-MB, LDH and AST) that are tightly bound to the contractile apparatus of the cardiomyocytes. Assessment of these cardiac enzymes is prerequisite in diabetic cardiomyopathy. Previous studies demonstrated increased levels of these biomarkers in diabetic animals [27]. In present study, the levels of these enzymes were decreased after ranolazine treatment suggesting halt of cardiac damage and offering cardio-protection.

The main mechanism by which STZ induces hyperglycaemia is generation of reactive oxygen species. These result in oxidative injury and apoptosis of pancreatic β cells, thus playing vital role in development of diabetic cardiomyopathy [19, 28]. In present study, STZ causes oxidative stress evident by increased MDA levels, an indicator of lipid peroxidation and reduced antioxidant defence systems. SOD is chief enzyme directly involved in eradication of ROS. Reduced glutathione is an intracellular radical scavenger while catalase breaks down peroxide molecules into water and oxygen. All of these enzyme levels were remarkably reduced in diabetic animals. The treatment with ranolazine withholds the changes in antioxidant defensive enzymes and lipid peroxidation. This beneficial effect of ranolazine against oxidative stress is helpful in managing lipid profile, cardiac enzymes and apoptosis of cardiomyocytes.

Many studies have shown handling intracellular calcium plays a pivotal role in cardiac function. One of the most effective mechanisms to maintain intracellular calcium homeostasis is by restoring it in sarcoendoplasmic reticulum with the help of SERCA pump. In diabetic heart due to oxidative stress, these pumps are damaged affecting contractility of the heart and contribute to altered relaxation [24, 25]. Consistent to this, in present study, the expression of SERCA were reduced in diabetic heart. Upregulating expression of SERCA pump by ranolazine treatment rescued contractile function in diseased hearts which might be due to increase in insulin sensitivity and secretion. The direct link between insulin levels and increase of SERCA has been reported in various studies [29].

Several histopathological studies showed mild endo-myocardial necrosis at 8 weeks and a severe focal endo-myocardial necrosis after 12 weeks in diabetic heart [26]. In present study, diabetic heart showed necrosis, disorganization, and degeneration of myofibers and vacuole formation as compared to non-diabetic heart whereas ranolazine treated group offered protection to heart at cellular level with minimal pathological changes like swelling of heart muscle fibres. The STZ treated animals showed disruption and shrinkage of islet cells of pancreas and disrupted pancreatic acinar cells as compared to normal control animals. The beneficial effect of ranolazine treatment was seen on microscopy of pancreas with intact exocrine and endocrine cells. The effect might be due to reduction in oxidative stress and improved glycaemic control.

4. CONCLUSION

In conclusion, the current study successfully replicates anti-diabetic effects of ranolazine observed in various studies. Ranolazine efficiently tackled hyperglycaemia induced changes in lipid profile, heart rhythm, LV function, cardiac enzyme levels, oxidative stress and morphology of heart. The beneficial effect of ranolazine is associated with amplification of SERCA levels. These results hold special value for developing strategies to combat diabetic cardiomyopathy.

5. MATERIALS AND METHODS

5.1. Drugs and chemicals

Streptozotocin (STZ), bovine albumin, epinephrine, thiobarbituric acid (TBA), trichloroacetic acid (TCA), tris buffer, 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman's Reagent), 5-thionitrobenzoic acid (TNB), TRIazol, used in this project were of analytical grade and were obtained from Sigma Aldrich Pvt. Ltd, Hyderabad. Ethylenediaminetetraacetic acid (EDTA), Carboxy methyl cellulose (CMC), sodium citrate, citric acid, potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), potassium chloride (KCl), and glucose were of analytical grade and were obtained from Merck Pvt. Ltd, Hyderabad. Calcium chloride ($CaCl_2$), magnesium sulphate ($MgSO_4$), sodium chloride (NaCl), and sodium pyruvate were of analytical grade and were obtained from Chemdyes Cop., Rajkot. All the biochemical tests had been performed using commercially available kits purchased from i-Chem, Jeev Diagnostic Pvt. Ltd, Chennai, India. Primers were purchased from Eurofins Genomics, Bangalore. qRT-PCR Kit was purchased from Applied Biosystems, Thermoscientific Inc., Ahmedabad. Ranolazine was procured from Torrent Research Centre, Ahmedabad (as gift sample).

Caution: The following chemicals are hazardous and should be handled carefully; trichloroacetic acid (TCA), 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman's Reagent), TRIazol. Follow safe procedures while handling these chemical substances.

5.2. Experimental animals and research protocol approval

Healthy male Sprague Dawley rats (180-220 g) were procured at our institutional animal house from Zydus Research Centre, Ahmedabad and housed in a group of 3 rats per cage under well-controlled conditions (Temperature: 22 ± 2°C, humidity: 55 ± 5 % and 12h /12h light-dark cycle). The animals had free access to conventional laboratory diet (purchased from Pranav Argo Pvt. Ltd) and water *ad libitum*.

The protocol for the experiment had been approved by Institutional Animal Ethical Committee (IAEC) 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. The protocol number for the project is APC/2018-IAEC/1848.

5.3. Dose selection and preparation of drug solution

Streptozotocin (60mg/kg) was prepared by dissolving it in a 0.1 M cold citrate buffer, pH 4.5 [30]. Ranolazine was freshly prepared daily by suspending in 0.5% carboxy methyl cellulose (CMC). The dose of ranolazine (40mg/kg) was fixed on basis of previous anti-diabetic study [31].

5.4. Induction of diabetic cardiomyopathy

The animals were fasted overnight and single intraperitoneal (i.p.) injection of freshly prepared STZ was given to induce diabetes. Hyperglycaemia was confirmed after 48 hours of injection by measuring the fasting blood glucose level using the glucose estimation kit. The rats with blood glucose level greater than 250 mg/dl were considered being diabetic and were included in the study [30].

5.5. Experimental protocol

The diabetic animals were randomly allocated in 3 experimental groups (10 animals per group) as follows: The normal control animals received distilled water and regular rat chow diet. The model control received single STZ injection (60mg/kg b.w., i.p.). The treatment group received single STZ injection (60mg/kg b.w., i.p.) + ranolazine (40 mg/kg b.w., p.o.) for 12 weeks.

During experimental period, body weight was measured weekly and expressed as growth rate using Eq. 1 [32]:

$$\text{Growth Rate} = \left(\frac{\text{Final Body Weight}}{\text{Initial Body weight}}\right)^{\left(\frac{1}{n-1}\right)} - 1 \qquad \text{[Eq. 1]}$$

At the end of the experimental period, ECG was measured under anaesthetized condition (Ketamine (50 mg/kg) + Xylazine (10 mg/kg)) using a Biopac Student Lab (MP-36 Biopac Systems, Inc., USA) and ten leads I, II, III, aVR, aVL, aVF and precordial VM (instead of V1, V2, V3), V4, V5, and V6. The type of alterations *viz*. QT prolongation, ST segment elevation or depression, and T wave inversion were considered [33].

Blood samples were collected and serum was separated for estimation of various biochemical parameters (*viz*, Glucose, CK-MB, LDH, AST, Total cholesterol, HDL Cholesterol and Triglyceride) by autoanalyzer (Turbochem 100, USA) using commercially available kits, according to manufacturer's instructions. LDL Cholesterol (Eq. 2), [34] VLDL (Eq. 3), [34] Cardiovascular risk indices (Eq. 4 & 5) [35] and atherogenic index of plasma (AIP) (Eq. 6) [35] were measured according to following formula:

$$\text{LDL cholesterol} = \text{Total cholesterol} - \left(\frac{\text{Triglycerides}}{5} + \text{HDL cholesterol}\right) \quad \text{[Eq. 2]}$$

$$\text{VLDL} = \frac{\text{Triglycerides}}{5} \quad \text{[Eq. 3]}$$

$$\text{Cardiovascular risk index 1} = \frac{\text{Total cholesterol}}{\text{HDL cholesterol}} \quad \text{[Eq. 4]}$$

$$\text{Cardiovascular risk index 2} = \frac{\text{LDL cholesterol}}{\text{HDL cholesterol}} \quad \text{[Eq. 5]}$$

$$\text{AIP} = \log_{10}\left(\frac{\text{Triglycerides}}{\text{HDL cholesterol}}\right) \quad \text{[Eq. 6]}$$

Animals were euthanized humanely for isolated heart preparation, oxidative stress parameters, SERCA expression levels and histopathological analysis.

5.6. Langendorff isolated perfused heart preparation

Perfusion of isolated hearts was performed according to the Langendorff technique [36]. The hearts were excised after thoracotomy and tied to the aortic cannula. Hearts were perfused with modified Krebs-Henseleit buffer (composition: $CaCl_2$ (1.5 mM), KCl (4.7 mM), KH_2PO_4 (1.18 mM), $MgSO_4$ (1.66 mM), NaCl (118 mM), $NaHCO_3$ (24.88 mM), glucose (5.55 mM), Na-pyruvate (2 mM), and bovine albumin (0.1%w/v)). The buffer was filtered by 0.45 µm membrane filter before use. The cannulated heart was rapidly connected with the Langendorff perfusion apparatus (flow rate of buffer: 9.7 ± 0.5 ml/min; carbogen (95% O_2 and 5% CO_2), and temperature: 37˚C). A latex balloon filled with 50% methanol was tied to the end of a polyethylene tube connected to the pressure transducer and was inserted into a left ventricle of the isolated heart. The diastolic pressure of 5 to 6 mmHg was adjusted and after 30 minutes, various parameters were measured.

Parameters measured included dP/dt_{max} (rate of maximum LV pressure rise); dP/dt_{min} (rate of minimum LV pressure fall) and Left ventricular end-diastolic pressure (LVEDP) as measurements of relaxation. The data was recorded by physiological recording system and Biopac recording device (MP-36 Biopac Systems, Inc., USA).

5.7. Oxidative stress parameters

The isolated heart tissues were cut into small pieces and were homogenized in ice-cold Tris-hydrochloride buffer (0.1M, pH 7.4). The homogenate was centrifuged at 1000 × g for 10 min at 4˚C in a refrigerated centrifuge. As an indicator of lipid peroxidation, level of Malondialdehyde was measured in supernatant by method of Slater and Sawyer [37]. The antioxidant enzyme levels were measured i.e. GSH, SOD and Catalase by method of Moron et al [38], Misra and Fridovich [39], and Aebi et al [40] respectively. Lowry's method was used to determine protein concentrations [41].

5.8. Quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted from heart tissue using TRIzol reagent. Both cDNA synthesis and PCR were performed simultaneously by using F416 L SYBR green qRT-PCR Kit. Quantitative RT-PCR was carried out using QuantStudio™ 5 Real-Time PCR system (Applied Biosystems, Foster, California, USA). The housekeeping gene encoding β-Actin was used as internal control. Relative expression was calculated using comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) [42].

5.9. Histopathology

Hearts and pancreas were fixed in 10% formalin, embedded in paraffin and 4 µm thick sections were cut. Deparaffinization of these sections was performed before staining them with haematoxylin and eosin to determine morphology [27].

6. STATISTICAL ANALYSIS

All the data were expressed as mean ± SEM. The results were compared using a computer based program (Prism, GraphPad version 6.01, GraphPad Software, Inc.). The significant difference between biochemical, LV function parameters, oxidative stress markers, SERCA levels of different groups were analysed using one-way ANOVA followed by post hoc Dunnett's test with *P* value <0.05. Growth Rate was analysed using two-way ANOVA followed by post hoc Bonferroni's test with *P* value <0.05.

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Conflict of interest statement: The authors declare that there are no conflicts of interest.

Ethics committee approval: The experimental protocol was approved by Institutional Animal Ethical Committee of Anand Pharmacy College, Anand, Gujarat, India, having protocol number (APC/2018-IAEC/1848) on October 3, 2018.

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