

## Original Article

# Eplerenone attenuates myocardial infarction in diabetic rats via modulation of the PI3K-Akt pathway and phosphorylation of GSK-3 $\beta$

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**Abstract:** We investigated the effect of eplerenone on myocardial infarcted diabetic rats via modulation of the PI3K/Akt pathway and its downstream target GSK-3 $\beta$ . Diabetes was induced by administration of a single dose of streptozotocin (55 mg/kg IP). Diabetic rats received either eplerenone or PI3K/Akt antagonist (wortmannin) or in combination for 14 days with concurrent administration of isoproterenol (100 mg/kg s.c) on 13<sup>th</sup> and 14<sup>th</sup> day. Isoproterenol prompted cardiotoxicity and was demonstrated by a decrease in the maximal positive rate of developed left ventricular pressure, the maximal negative rate of developed left ventricular pressure and an increase in left ventricular end-diastolic pressure along with oxidative stress. Myocardial infarcted diabetic rats exhibited increased myonecrosis, edema, and apoptotic cell death. Treatment with eplerenone significantly improved the redox status of the myocardium. Eplerenone markedly inhibited Bax expression, TUNEL-positive cells, and myonecrosis. On the other hand, the administration of eplerenone and wortmanin did not draw out the same effects, when administered concomitantly or individually. Moreover, the rats treated with eplerenone showed increased expression of PI3K/Akt and decreased its downstream target GSK-3 $\beta$ . The present study confirms the protective effects of eplerenone on myocardial infarction in diabetic rats via modulation of PI3K/Akt pathway and its downstream regulator GSK-3 $\beta$ .

**Keywords:** STZ, ISO, PI3K/Akt/GSK-3 $\beta$ , eplerenone, diabetes

## Introduction

Myocardial infarction is extensively built up in patients with diabetes mellitus, foremost to augmented mortality and morbidity levels. Oxidative stress and cardiac apoptosis have been identified as root causes of diabetes-induced cardiovascular complications [1, 2]. Whereas, Myocardial infarction in current scenario generates billions of dollars in healthcare costs globally and leads to fear-provoking round about all the countries [3]. It has been well proven that increased in the generation of superoxide anions and reactive oxygen species is in-line with diabetic complications arising in humans as well as in animals [4, 5]. On the whole, the treatments accessible for ischemic

injury, including myocardial infarction are focussed toward reinstatement of blood supply to ischemic tissue and preventing the damage inflicted at the injury [6].

Isoproterenol, a synthetic non-selective  $\beta$ -adrenoceptor agonist, has been recognized to provoke myocardial infarction in rats as a result of distressed physiological equilibrium between production of free radicals and antioxidative defense system [7]. The pathophysiological and morphological changes coupled with isoproterenol in rats are analogous to those observed for human myocardial infarction [8].

Recently, much progress has been made in elucidating the signal transduction pathways

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involve in the cardioprotection in relation to convey the extracellular signal initiated by the stimuli to the intracellular targets of cardioprotection, with many of these pathways involving the activation of a diverse array of survival protein kinase cascades. Among the known cell survival pathways, the PI-3-kinase (phosphatidylinositol 3-kinase)/Akt pathways are of considerable importance [9]. Activation of PI3K/Akt pathway prevents ROS-induced apoptosis in human umbilical vein endothelial cells [10]. In relation to the PI3K/Akt pathway, the glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) has been revealed to promote apoptosis and turn on apoptosis-related protein kinase, caspases proteins, and Bax gene responsible for apoptosis, led to produces apoptosis [11]. It has been already reported that GSK-3 $\beta$  is regulated by PI3K/Akt signal transduction pathway [12].

Eplerenone, a selective aldosterone antagonist, is a renowned remedy used to treat heart failure and related complications [13]. Though, the soundness of its beneficial effect in diabetic cardiac complications has not been explored yet. Pre-clinical evidences put forward that aldosterone can have an undesirable effect on the myocardium. Previous reports suggests that blockade of aldosterone shown to have reduced oxidative stress in atherosclerosis [14]. Moreover, recent studies revealed that eplerenone activates the PI3K/Akt signalling pathway [15]. Owing to this, activation of Akt leads to downstream the GSK-3 $\beta$ . Hence, in view of this fact, the present study was designed to explore, the effect of eplerenone via modulation of PI3K-Akt and phosphorylation of its downstream target GSK-3 $\beta$  pathway to affect cardiac function, cardiac injury markers, endogenous antioxidants, and tissue architecture in diabetic rats.

### Materials and methods

#### Animals

The Male Wistar rats, (200-250 g) were kept at standard laboratory conditions under natural light and dark cycles, humidity (60 $\pm$ 10%) and a constant room temperature (25 $\pm$ 5 $^{\circ}$ C). The study protocol was approved by the Institutional Animal Ethics Committee (Approval No. IAEC/CPCSEA/RCPIPER/2014-17) of R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, India and conforms to the

Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Reg. No. 651/02/c/CPCSEA).

#### Chemicals

Eplerenone was obtained as a gift sample from Glenmark laboratories, Nashik, India. Isoproterenol was Purchased from Sigma Aldrich, USA. The ABC staining kit and primary antibodies like Bax mouse monoclonal IgG2b, Bcl-2 mouse monoclonal IgG1, PI3K (SC-1637, LotK1910), AKT (SC-81434, Lot J3014) and GSK3  $\beta$  (SC-81462 Lot-10511) were procured from Santa Cruz Biotechnology, USA.

#### Experimental protocol

**Induction of diabetes:** Diabetes was induced by a single intraperitoneal injection of streptozotocin [16] (STZ) (55 mg/kg body weight) dissolved in 0.1 M cold citrate buffer (pH 4.5). Diabetes was confirmed 3 days of post-injection of STZ by estimating the serum glucose level. Rats with blood glucose level higher than 250 mg/dl were considered diabetic and included in the study protocol.

**Induction of myocardial infarction:** On the days 13<sup>th</sup> and 14<sup>th</sup> of study protocol, isoproterenol (ISO) (100 mg/kg) was injected subcutaneously to rats with 24 h of interval to induce experimental myocardial infarction.

#### Experimental design

After confirming the onset of diabetes using serum glucose level, rats were randomly divided into five different groups (n=14).

**Group I diabetic control:** Rats were treated with distilled water (1 ml/kg/day p.o.) for 14 days and on the day 13<sup>th</sup> and 14<sup>th</sup> they were treated with 0.3 ml of saline at the interval of 24 h.

**Group II diabetic isoproterenol:** Rats were administered water orally for 14 days along with concurrent administration of ISO (100 mg/kg, s.c.at the interval of 24 h) on the day 13<sup>th</sup> and 14<sup>th</sup>.

**Group III diabetic isoproterenol rats treated with eplerenone:** Animals were treated with eplerenone (150 mg/kg/day) orally for a period of 14 days along with concurrent administration of ISO on the day 13<sup>th</sup> and 14<sup>th</sup>.

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**Table 1.** Effect of eplerenone on changes in heart weight, blood glucose, body weight and heart weight to body weight ration

Treatment groups (All diabetic)	Heart weight (g)	Blood glucose (mg/dl)	BW (g)	HW/BW ratio (mg/g)
Control	0.69±0.04	490.8±53.6	245±3.9	2.816±0.0102
ISO	0.54±0.06 <sup>#</sup>	506.5±50.6 <sup>##</sup>	220±3.4 <sup>#</sup>	2.454±0.0176
EPL	0.61±0.07 <sup>*</sup>	490.5±86.7 <sup>*</sup>	244±2.7 <sup>*</sup>	2.4796±0.025
Wort	0.45±0.09	524.3±77.1	208±3.6	2.163±0.0241
Wort + EPL	0.58±0.04 <sup>§</sup>	505.5±75.46	226±4.1	2.566±0.0251

Data were expressed as the mean ± S. E. M. Significance was determined by one-way ANOVA (Analysis of variance) followed by the Bonferroni's post hoc test: <sup>\*</sup>P<0.05 as compared to diabetic ISO; <sup>#</sup>P<0.05, <sup>##</sup>P<0.01 as compared to diabetic control; <sup>§</sup>P<0.001 as compared to eplerenone. (BW: body weight, HW: heart weight. All groups were diabetic ISO: Isoproterenol; EPL: Eplerenone; Wort: wortmannin; EPL + Wort: Eplerenone + wortmannin).

*Group V diabetic isoproterenol rats treated with wortmannin:* Animals were treated with wortmannin (1 mg/kg/day, i.p.) for a period of 14 days with concurrent administration of ISO on days 13<sup>th</sup> and 14<sup>th</sup> at the interval of 24 h.

*Group IV diabetic isoproterenol rats treated with wortmannin and eplerenone:* Animals were treated with wortmannin 15 min prior to administration of eplerenone for 14 days with concurrent administration of ISO on days 13<sup>th</sup> and 14<sup>th</sup> at the interval of 24 h.

The experimental animals were examined at regular intervals throughout the course of the study and any changes in body weight and/or food and water intake as well as mortality rate were recorded.

### Evaluation of parameters

*Surgical procedures for recording hemodynamic parameters:* In brief, rats were anesthetized with an intraperitoneal injection containing pentobarbitone sodium (60 mg/kg) and atropine (0.1 mg/kg) to lessen the Bronchotracheal discharge as well as to sustain the heart rate, mainly throughout the phase of surgery in all experimental groups. The surgery for measurement of hemodynamic parameters and left ventricular assessment were performed according to the method of Reddy *et al.*, (2015) [8].

*Estimation of cardiac injury markers and oxidative stress:* A 10% homogenate was used to estimate the amount of creatine kinase on the myocardial bundle (CK-MB) and Lactate dehydrogenase (LDH) [17]. The oxidative stress was estimated by analyzing the content of malondialdehyde (MDA) [18], reduced glutathione

(GSH) [19], Catalase [19] and activity of superoxide dismutase (SOD) [7].

### Determination of myocardial apoptosis

*Immunohistostaining for the tracing of Bax and Bcl-2 proteins:* Immunohistostaining for the identification of Bax and Bcl-2 proteins as an apoptotic markers was performed as previously described by Rani *et al.*, (2016) [20].

*TUNEL assay:* TUNEL assay was performed using a cell death detection kit (Bio Vision, Inc, USA) as described by Kocak *et al.*, (2016) [21] and according to the manufacturer's instructions.

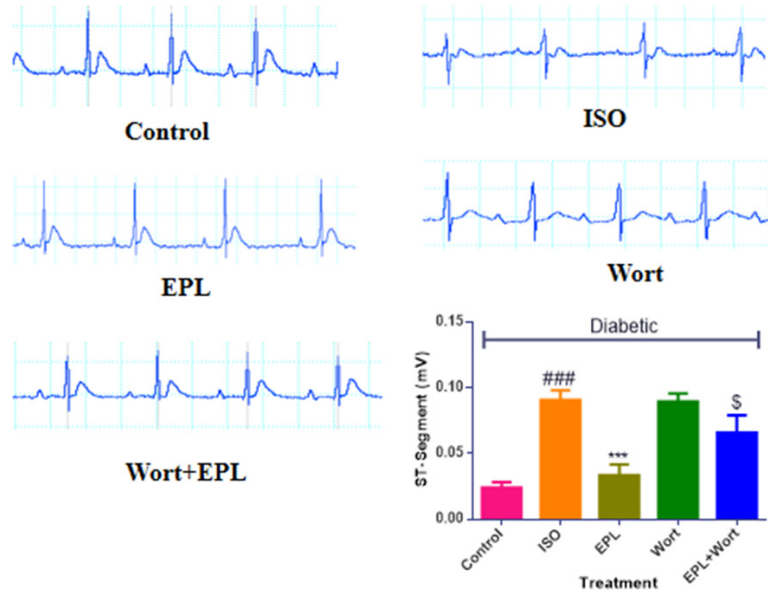
*Western blot analysis:* Myocardial tissue was homogenized in RIPA lysis buffer and protein was estimated. Western blot analysis was performed as described previously [22].

*Light microscopic evaluation:* Myocardial tissues were fixed in buffered formalin solution and embedded in paraffin. Serial sections (3 mm thick) were cut using microtome. Every section was stained with haematoxylin and eosin (H&E). Sections were examined under the light microscope (Nikon, Tokyo, Japan), and photographs were taken. The pathologist performing microscopy was blind to the treatment standing of the experimental subject.

### Statistical analysis

The data are expressed as mean ± SEM, statistical significances were analyzed by one-way ANOVA (Analysis of variance) followed by Bonferroni's post hoc test using a graph pad, prism software, version 6.0, USA. The value of P<0.05 was considered as a significant.

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**Figure 1.** Effect of EPL on cardiac function and ECG wave forms in STZ-isoproterenol challenged rats. All groups are diabetic, where ISO: Isoproterenol; EPL: Eplerenone; Wort: wortmannin; Wort + EPL: wortmannin + Eplerenone. Data were expressed as the mean  $\pm$  S.E.M. Significance was determined by one-way ANOVA (Analysis of variance) followed by the Bonferroni's post hoc test: \* $P < 0.05$ , \*\* $P < 0.01$  as compared to Diabetic ISO; ### $P < 0.001$  as compared to diabetic control; \$ $P < 0.001$  as compared to eplerenone.

### Results

#### Mortality

An on the whole mortality of 5% was observed throughout the study protocol. The animals were lost because of stern increase in diabetes, bleeding during surgery for recording of hemodynamic parameters.

#### General observations

Administration of STZ in animals increases blood glucose in entire experimental groups as compared to their basal readings. However, body weights, heart weight and heart to body weight ratio does not show any significant change in dissimilar experimental groups (Table 1).

#### Eplerenone normalised ECG waveforms in STZ-isoproterenol challenged rats

Diabetic rats treated with ISO showed alterations in ECG waveforms as a significant elevation ( $P < 0.01$ ) of the ST segment was observed as compared to diabetic control. While treatment with eplerenone at the dose of 150 mg/

kg showed normal wave formations in the ECG and significantly normalised the ST-segment as compared with diabetic ISO treated rats (Figure 1). The diabetic rats treated with wortmannin showed significant increase in the ST segment as well as deformations in the ECG wave formations as compared with diabetic isoproterenol treated rats (Figure 1).

*Eplerenone improved hemodynamic and ventricular dysfunction in STZ-isoproterenol challenged rats*

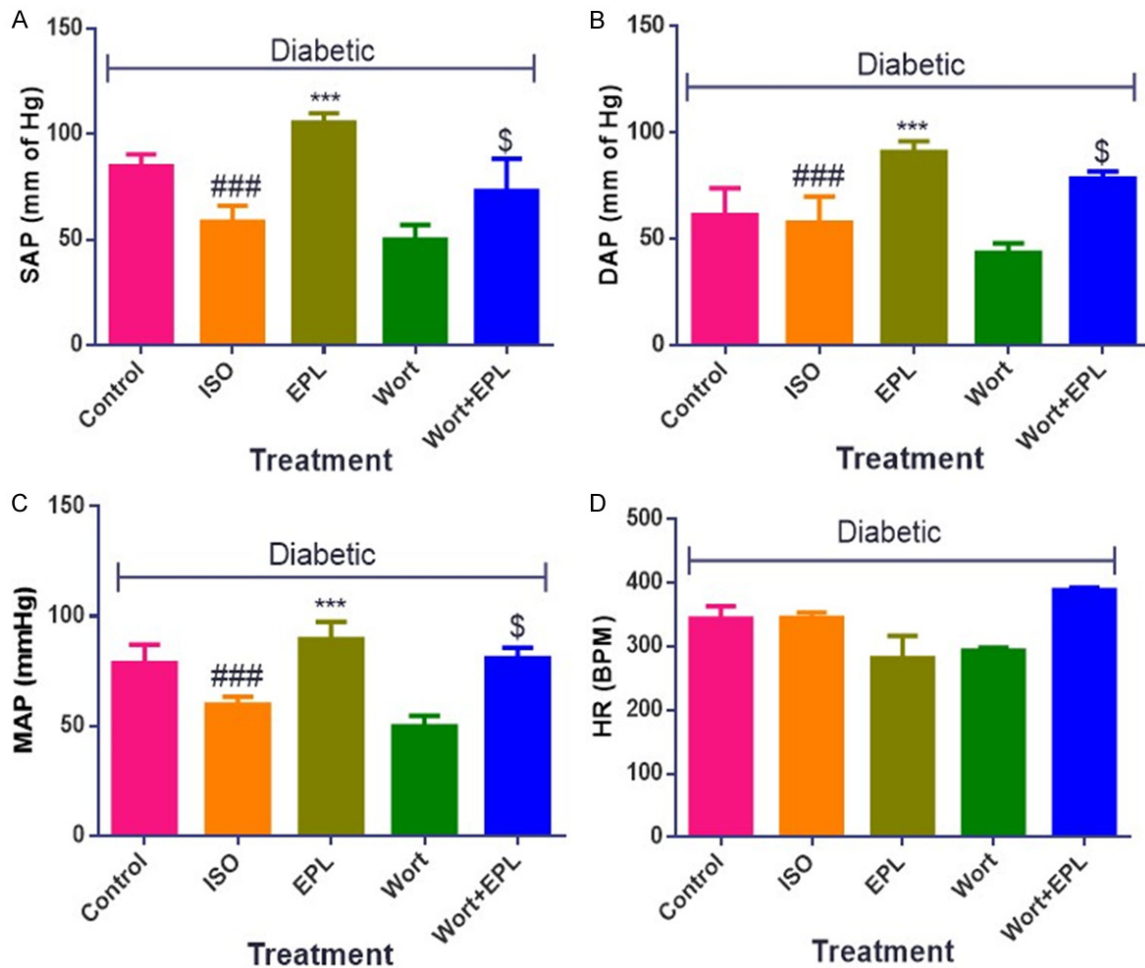
Diabetic ISO treated rats showed a significant decrease ( $P < 0.001$ ) in SAP, DAP and MAP as well as maximal positive and a negative rate of left ventricular pressure ( $\pm$ LVdP/dtmax), and

increase in left ventricular end-diastolic pressure (LVEDP) as compared to the diabetic control rats. Treatment of eplerenone for fourteen days normalised hemodynamic parameters and left ventricular assessment functions with respect to the diabetic ISO rats (Figures 2 and 3). Administration of wortmannin to diabetic myocardial infarcted rats does not attenuated the SAP, DAP and MAP, confirms the antagonistic activity of PI3K.

#### *Eplerenone restored the oxidative stress in STZ-isoproterenol challenged rats*

Diabetic ISO challenged rats showed noteworthy boost in the oxidative stress as a significant increase ( $P < 0.001$ ) in malondialdehyde content as well as a decrease in the levels of GSH, SOD and catalase ( $P < 0.001$ ) in tissue homogenate. Treatment with eplerenone showed contrast results that obtained with diabetic ISO challenged rats as it normalised the level of MDA as well as significantly increase ( $P < 0.001$ ) the levels of endogenous antioxidants in respect to reduce the oxidative stress, while treatment with the wortmannin alone or in combination with eplerenone showed increased in the oxidative stress (Table 2).

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**Figure 2.** Effects of EPL on hemodynamic parameters in STZ-isoproterenol challenged rats. A. Systolic arterial pressure (SAP). B. Diastolic arterial pressure (DAP). C. Mean arterial pressure (MAP). D. Heart Rate. Data were expressed as the mean  $\pm$  S.E.M. Significance was determined by one-way ANOVA (Analysis of variance) followed by the Bonferroni's post hoc test: \*\*\* $P < 0.001$  as compared to Diabetic ISO; ### $P < 0.001$  as compared to diabetic control; \$ $P < 0.001$  as compared to eplerenone. (All the animals from groups were diabetic, where ISO: Isoproterenol; EPL: Eplerenone; Wort: wortmannin; Wort + EPL: wortmanin + Eplerenone).

### *Eplerenone recovered cardiac injury markers in STZ-isoproterenol challenged rats*

Diabetic ISO challenged rats showed significant decreased ( $P < 0.001$ ) in the activities of CK-MB and LDH. Treatment with eplerenone showed a significant attenuation ( $P < 0.001$ ) of CK-MB and LDH showed positive effect and maintained the myocardial membrane integrity as compared with the diabetic ISO treated rats (Figure 4).

### *Eplerenone amplified PI3K, Akt expression while repressed the GSK-3 $\beta$ expression in STZ-isoproterenol challenged rats*

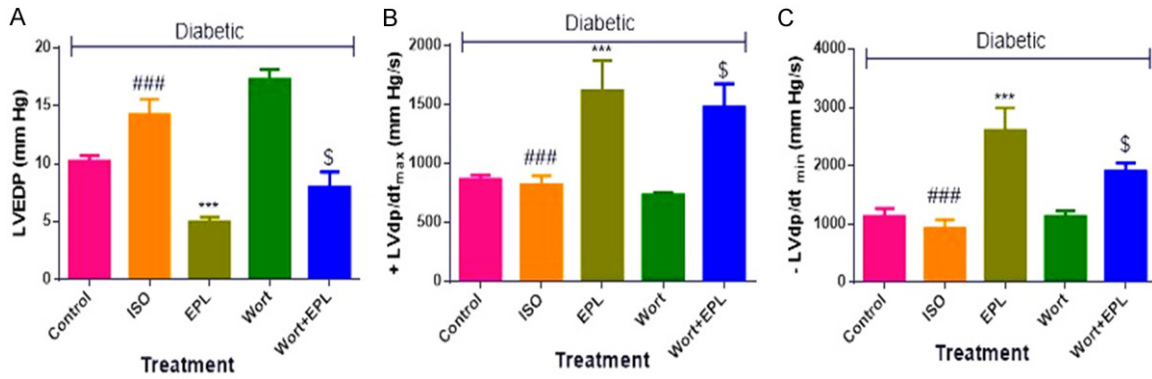
The rats challenged with diabetic ISO showed significant decrease in the expression of PI3K and Akt ( $P < 0.001$ ) while amplify the expression

of GSK-3 $\beta$  ( $P < 0.001$ ) in myocardial tissue homogenates as shown in (Figure 5). Fourteen days treatment with the eplerenone significantly modulates the protein expression and phosphorylates PI3K and Akt ( $P < 0.001$ ), however significantly depressed ( $P < 0.001$ ) GSK-3 $\beta$  expression. The diabetic ISO rats treated with wortmannin showed inhibition of PI3K/Akt, Thus, the results obtained confirms the PI3K modulatory and subsequent GSK-3 $\beta$  inhibitory activity of eplerenone in diabetic ISO challenged rats (Figure 5).

### *Eplerenone reduced Bax and increased Bcl-2 expression in STZ-isoproterenol challenged rats*

In comparison to diabetic control rats, the rats challenged with ISO showed significant increase

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**Figure 3.** Effects of EPL on peak positive and negative pressure development in STZ-isoproterenol challenged rats. A. Left ventricular end diastolic pressure (LVEDP). B. Maximal positive rate of left ventricular pressure (+LVdP/dt<sub>max</sub>). C. Maximal negative rate of left ventricular pressure (-LVdP/dt<sub>min</sub>). Data were expressed as the mean  $\pm$  S.E.M. Significance was determined by one-way ANOVA (Analysis of variance) followed by the Bonferroni's post hoc test: \*\*\*P<0.001 as compared to Diabetic ISO; ###p<0.001 as compared to diabetic control; \$P<0.001 as compared to eplerenone. (All the animals from groups were diabetic, where ISO: Isoproterenol; EPL: Eplerenone; Wort: wortmannin; Wort + EPL: wortmanin + Eplerenone).

**Table 2.** Effect of eplerenone on oxidative stress in streptozotocin-isoproterenol challenged rats

Treatment groups (All diabetic)	MDA ( $\mu\text{g}/\text{mg}$ of protein)	GSH ( $\mu\text{g}/\text{mg}$ of protein)	SOD (U/mg of protein)	Catalase (U/mg of protein)
Control	92.90 $\pm$ 12.89	0.999 $\pm$ 0.013	22.23 $\pm$ 1.23	28.63 $\pm$ 0.36
ISO	267.1 $\pm$ 14.15###	0.373 $\pm$ 0.153###	3.62 $\pm$ 0.98###	2.65 $\pm$ 0.03###
EPL	144.4 $\pm$ 15.34***	0.937 $\pm$ 0.029***	18.36 $\pm$ 1.26***	21.36 $\pm$ 0.41***
Wort	253.2 $\pm$ 10.23	0.350 $\pm$ 0.035	5.32 $\pm$ 0.35	6.36 $\pm$ 0.09
Wort + EPL	105.3 $\pm$ 21.33 <sup>s</sup>	0.610 $\pm$ 0.056 <sup>s</sup>	12.36 $\pm$ 1.03 <sup>s</sup>	18.56 $\pm$ 0.19 <sup>s</sup>

Data were expressed as the mean  $\pm$  S.E.M. Significance was determined by one-way ANOV (Analysis of variance) followed by the Bonferroni's post hoc test: \*\*\*p<0.001 as compared to Diabetic ISO; ###p<0.001 as compared to diabetic control; <sup>s</sup>p<0.001 as compared to eplerenone. (All groups were diabetic ISO: Isoproterenol; EPL: Eplerenone; Wort: wortmannin; EPL + Wort: Eplerenone + wortmanin).

in the Bax and decreased Bcl2 expression showed augmented in the apoptosis, while treatment with the eplerenone showed decreased in the protein expression of Bax and increased Bcl2. The rat treated with wortmanin does not show any significant change in the expression of the Bax and Bcl2 (**Figure 6A-J**).

Further, to support the role of eplerenone on apoptosis, TUNEL assay was performed to detect DNA fragmentation in apoptotic nuclei. An increase in the number of TUNEL positive nuclei was observed in diabetic ISO challenged rats myocardium. On the contrary, few TUNEL positive apoptotic nuclei were seen in eplerenone treatment group (**Figure 6K-O**).

### *Eplerenone conserved myocardium arrangement in STZ-isoproterenol challenged rats*

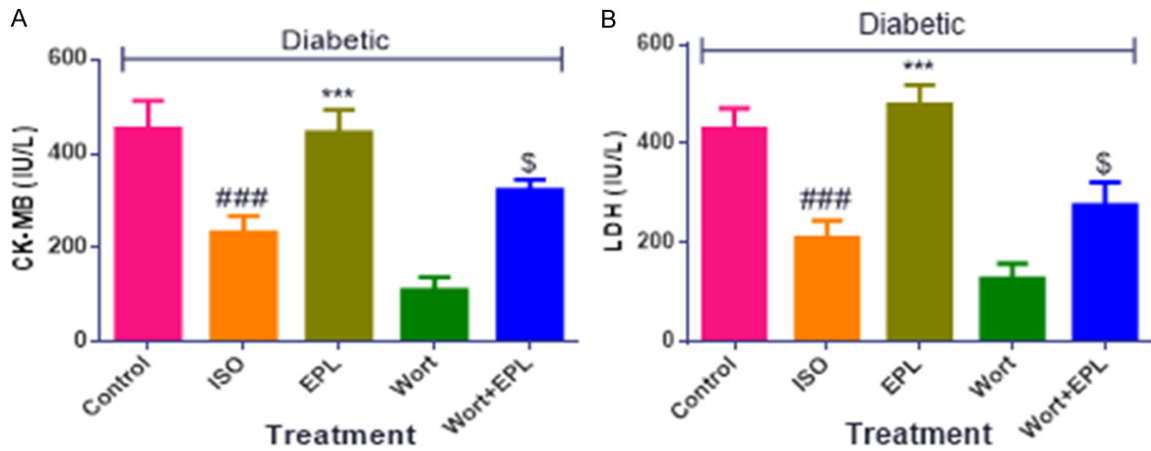
Diabetic control rats exhibited normal myocardial architecture with no evidence of inflamma-

tion and edema. Diabetic ISO injured myocardium displayed marked edema and membrane damage along with infiltration of inflammatory cells and higher histological score. Intriguingly, treatment with eplerenone improved myonecrosis, preserved myocardial architecture, and exhibited a low histological injury score. The rats treated with wortmanin alone or combination with eplerenone showed increased inflammation and increases the histological scores (**Figure 7** and **Table 3**).

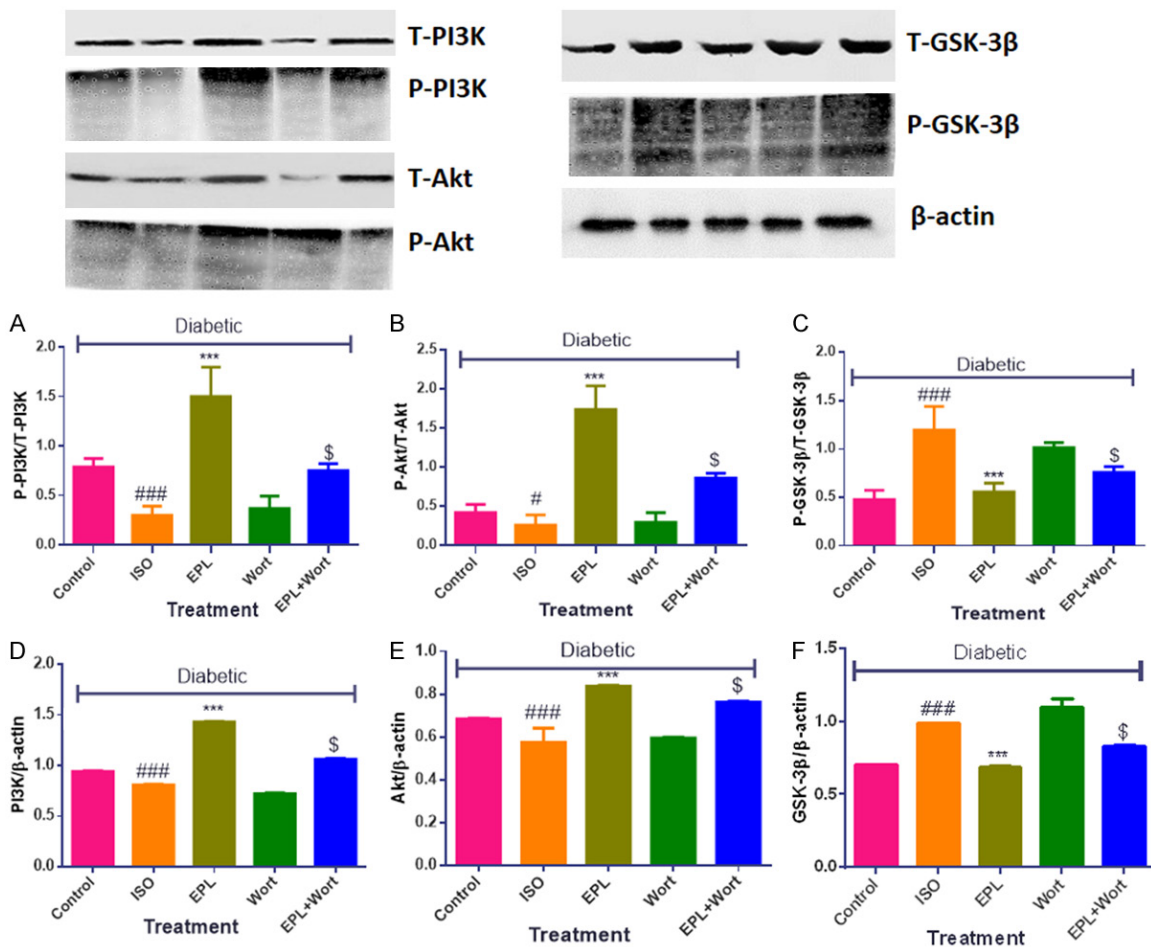
### Discussion

The present study for the first time evidenced the involvement of PI3K/Akt and there downstream GSK-3 $\beta$  pathway in the diabetic myocardial infarction. In this, streptozotocin was used for the orientation of diabetes in rats, seeing as it enters the  $\beta$ -cells of pancreas and alkylates DNA. This diminishes the activity of NAD<sup>+</sup> as

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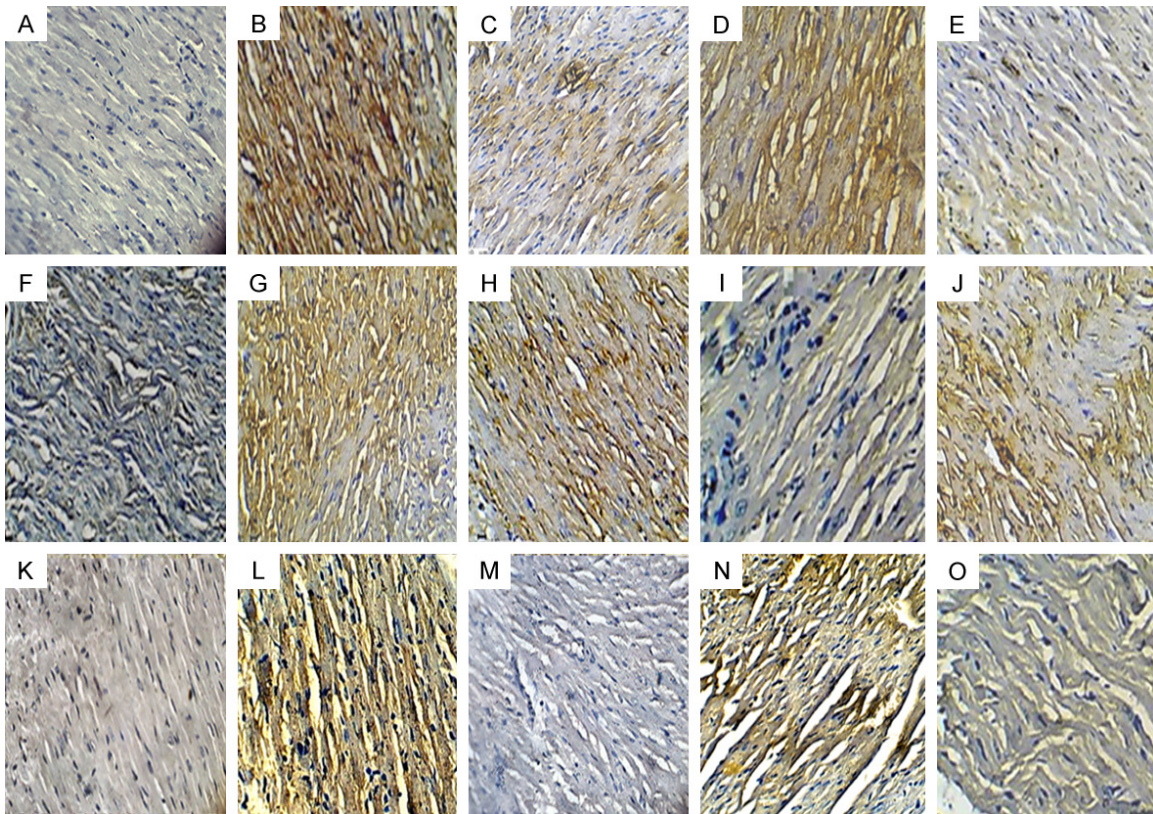
**Figure 4.** Effects of EPL on cardiac injury markers in STZ-isoproterenol challenged rats. A. Creatine kinase-MB (CK-MB), B. Lactate dehydrogenase (LDH). Data were expressed as the mean  $\pm$  S.E.M. Significance was determined by one-way ANOVA (Analysis of variance) followed by the Bonferroni's post hoc test: \*\*\* $P < 0.001$  as compared to Diabetic ISO; ### $P < 0.001$  as compared to diabetic control; \$ $P < 0.001$  as compared to eplerenone. (All the animals from groups were diabetic, where ISO: Isoproterenol; EPL: Eplerenone; Wort: wortmannin; Wort + EPL: wortmanin + Eplerenone).



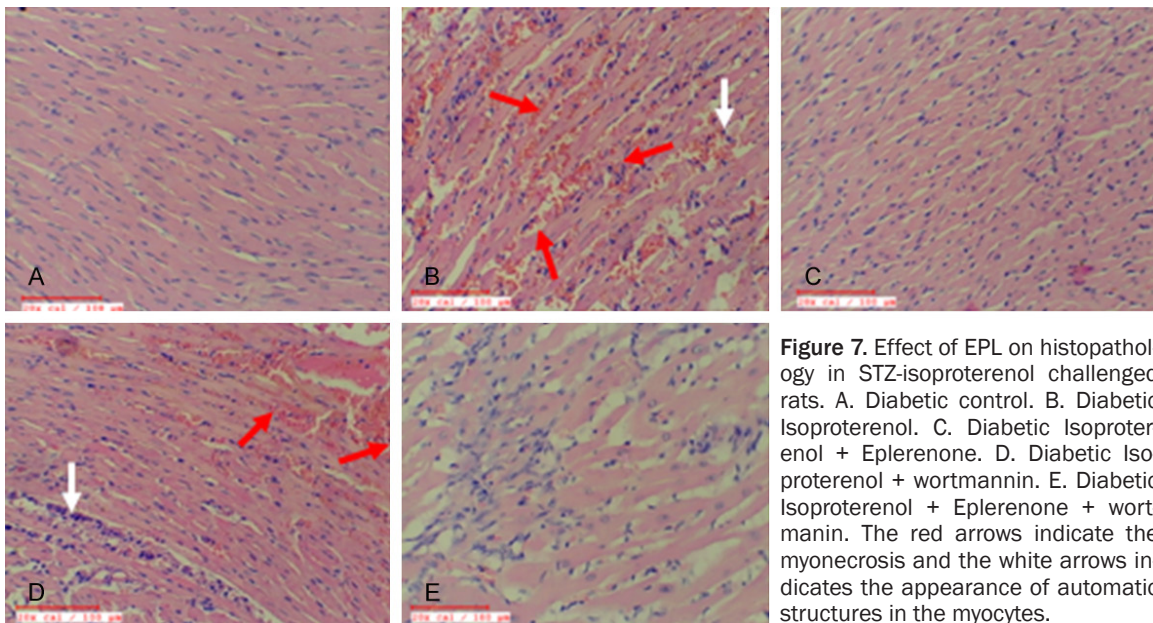
**Figure 5.** Effects of EPL on PI3K, Akt and GSK-3β expression in STZ-isoproterenol challenged rats. (A) Ratio of P-PI3K to T-PI3K, (B) Ratio of P-Akt to T-Akt, (C) Ratio of P-GSK-3β to T-GSK-3β, (D) Ratio of PI3K to β-actin, (E) Ratio of Akt to β-actin, (F) Ratio of GSK-3β to β-actin. Data were expressed as the mean  $\pm$  S.E.M. Significance was determined by one-way ANOVA (Analysis of variance) followed by the Bonferroni's post hoc test: \*\*\* $P < 0.001$  as

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compared to Diabetic ISO; ###P<0.001 as compared to diabetic control; \$P<0.001 as compared to eplerenone. (All the animals from groups were diabetic, where ISO: Isoproterenol; EPL: Eplerenone; Wort: wortmannin; Wort + EPL: wortmanin + Eplerenone).



**Figure 6.** Effects of EPL on expression of Bcl-2, Bax proteins and TUNEL-positive cells in STZ-isoproterenol challenged rats. Immunohistochemical analysis of Bax (A-E) and Bcl-2 (F-J); TUNEL Positive cell (K-O; 20X; scale bar 100 μm) in various groups. (A, F, K) Diabetic control; (B, G, L) Diabetic + isoproterenol; (C, H, M): Diabetic Isoproterenol + Eplerenone; (D, I, N) Diabetic isoproterenol + wortmannin (E, J, O): Diabetic Isoproterenol + Eplerenone + wortmannin.



**Figure 7.** Effect of EPL on histopathology in STZ-isoproterenol challenged rats. A. Diabetic control. B. Diabetic Isoproterenol. C. Diabetic Isoproterenol + Eplerenone. D. Diabetic Isoproterenol + wortmannin. E. Diabetic Isoproterenol + Eplerenone + wortmannin. The red arrows indicate the myonecrosis and the white arrows indicates the appearance of automatic structures in the myocytes.



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**Table 3.** Effect of Eplerenone on histopathological changes in different experimental groups

Treatment groups (All diabetic)	Myonecrosis	Inflammatory cells	Edema
Control	-	-	-
ISO	++	++	+
EPL	-	-	-
Wort	+++	++	+++
Wort + EPL	+	+	-

Severe (+++), moderate (++) , less (+), absent (-). All groups were diabetic ISO: Isoproterenol; EPL: Eplerenone; Wort: wortmannin; EPL+ Wort: Eplerenone + wortmannin.

well as ATP and exaggerates the diabetic condition similar in the humans by activating poly ADP-ribosylation pathway [23, 24].

Administration of subcutaneous injections of ISO causes imbalance between oxygen supply and demand by the myocytes from end to end increasing the chronotropism and inotropism important to explicit myocardial function and amplify in the calcium overload in the myocardium [7]. The present study deals to establish, the effects of eplerenone in treatment of diabetic cardiac complication in animals by exhibiting improved cardiac functionalities, boosting endogenous defense systems, reducing myocardial apoptosis, preserving myofibril structure and morphology through its modulatory effect on PI3K/Akt pathway and downstream target GSK-3 $\beta$ . In the present study, we employed wortmannin as PI3K/Akt antagonist to build the confirmation of modulatory activity of eplerenone.

Previously, it has been described that administration of isoproterenol in rats associated with the myocardial structural injury. Which eventually emerges in perturbed hemodynamic and contractile dysfunction [25], alteration in the hemodynamic parameters as evidenced by decrease in the systolic, diastolic and mean arterial blood pressure, also significantly impaired inotropic and lusitropic state ( $\pm$ LVdP/dtmax), and increased ventricular remodelling (preload LVEDP). While treatment with eplerenone improved LVEDP by escalating inotropic (+LVdP/dtmax, a marker of myocardial contraction) and lusitropic (-LVdP/dtmax, a marker of myocardial relaxation) circumstances of myocardium, PI3k/Akt antagonist wortmannin deteriorate the situation. Moreover, prior treatment of eplerenone than wortmannin also prevented

the rats from further toxicity of the isoproterenol. Modulation of PI3K/Akt pathway is shown to have beneficial effects in the cardiac injury as observed by Hua et al., (2007) [26]. Thus, these observations propose the protection of myocardium because of eplerenone in diabetic myocardial infarcted rats by means of PI3K/Akt pathway and so as to its activation may augment these effects.

Administration of isoproterenol in diabetic rats produces a myocardial excited function due to augmented chronotropism, inotropism and led to deleterious oxidative stress. These observations are in accordance with the previous literature [27]. In the present study, administration of eplerenone in diabetic myocardial infarcted rats showed decrease in the oxidative stress as observed by decreasing the malondialdehyde content as well as increasing the levels of endogenous antioxidant enzymes, while administration of wortmannin worsen the condition as compared with the diabetic control rats. The creatinine kinase-MB isoenzyme and lactate dehydrogenase are present in the myocardium and have been dilapidated as a forecaster for pathology and internal changes occurs in the myocardium [28]. In the current study, we observed that these enzymes were decreased in the myocardium of diabetic ISO rats, which is in line with the previous study reported by Zaitone et al. (2016) [29]. Treatment with eplerenone prevent the loss of membrane bound enzymes in diabetic myocardial infarcted rats, while treatment with the wortmannin does not prevent the loss of enzymes from the myocardium and confirms the involvement of PI3K/Akt in the protection of myocardial infarction. The present study demonstrated that administration of eplerenone in diabetic myocardial infarcted rats showed increased expression of PI3k, and Akt protein, also reduced the expression of GSK-3 $\beta$  through western blot analysis, whereas the rats treated with wortmannin significantly decreased the concentration of PI3K, Akt protein expression on account of the opposed possessions of wortmannin, thus the observation confirms the role of PI3k/Akt mediated pathway in the diabetic cardiac complications. Various reports have demonstrated the role of PI3K and Akt in different pathological conditions [30, 31] while, several additional confirmative studies may be required for further strengthening of this hypothesis. In addition to above parameters, myocardial apopto-

sis was also studied, which is recommended to be one of the chief pathological parameters getting worse in diabetic myocardial injury [32].

Chen et al. (2016) [33] reported that reducing the apoptosis signaling in the myocardium might be helpful to prevent the loss of contractile function of the myocardium, and decrease the chances of further damage to the myocardium. From the above report, we explored the fundamental mechanism responsible for the enhancement of myocardial utility following administration of eplerenone in diabetic cardiac complications, the expression of pro apoptotic protein Bax and anti-apoptotic protein Bcl-2 were performed using immunohistochemistry and TUNEL assay was executed, which could be employed for the detection of DNA fragmentation.

Diabetic myocardial infarcted rats significantly reduced Bcl-2 and increased Bax expression as well as TUNEL-positive cells. Furthermore, treatment with eplerenone in myocardial infarcted diabetic rats showed significant anti-apoptotic activity as represented by amplified Bcl-2 expression and dwindled expression of Bax also the TUNEL-positive cells. Though, administration of wortmanin deteriorates the situation in the myocardial infarcted diabetic rats. The involvement of PI3K/Akt pathway in the process of apoptosis is well established [34]. Thus, the reduction of eplerenone induced improvement in the diabetic myocardial infarction in presence of wortmanin confirms the involvement of PI3K/Akt pathway in the eplerenone mediated actions in diabetic myocardial infarcted rats. Further to confirm the salvaging effect of eplerenone in the myocardial infarcted diabetic rats light microscopy was performed, the rats subjected to diabetic myocardial infarction showed signs of infarction with Inflammatory cells, myonecrosis and edemetic structure. Eplerenone treatment in myocardial infarcted diabetic rats showed the defense of the regular morphology of myocardium with no signs of infarction, the opposite results were observed in the rats treated with wortmannin as an increase in the myonecrosis oedema and increased inflammatory cells.

### Conclusion

Taken together, all the studied parameters, it was concluded that eplerenone significantly

attenuates streptozotocin-isoproterenol induced myocardial infarction and the mechanism for this favorable effect could be explained by PI3K/Akt activation and downstream of its GSK-3 $\beta$ . Therefore, we have provided direct evidence that the PI3K/Akt and GSK-3 $\beta$  signalling pathway plays a significant role in regulating oxidative stress and subsequent tissue injury. This is the first study ever conducted to describe the cardio protective effect of eplerenone through the PI3K/Akt and its downstream target GSK3- $\beta$  in myocardial infarcted diabetic rats.

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### Disclosure of conflict of interest

None.

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