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The Cardioprotective Potential of Sitagliptin in Myocardial Ischemia Reperfusion Injury.

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ABSTRACT

Sitagliptinis an oral anti-hyperglycemic agent that belongs to the class of DPP-4 inhibitors. Nowadays, DPP-4 inhibitors are being investigated for their applicability in other pathological conditions, both in animal studies and in clinical settings. Based on the fact that diabetes mellitusincreases cardiovascular risk including myocardial infarction (MI) and that DPP-4 substrates have a role outside glucose regulation, thus the aim of the current study is to investigate the effect of sitagliptin on the myocardial injury induced in ischemic/reperfused rats' hearts and to investigate its effect on the apoptotic/anti-apoptotic and necrotic markers. Male Wistar rats were randomized into 3 groups, sham, I/R and sitagliptin (300 mg/kg, p.o) + I/R. Sitagliptin was administered for 3 days before induction of coronary I/R. Pretreatment with sitagliptin provided significant protection against I/R injury as it decreased the infarct size, suppressed the elevated ST segment heights, and reduced the cardiac enzymes LDH, CK-MB and cTn-I.. On the molecular level, sitagliptin up regulated the mRNA levels of Bcl2 and down regulated that of Bax and cypD. In conclusion, sitagliptin have the ability to attenuate myocardial I/R injury in rats through interfering with the apoptoticand necrotic pathways.
Keywords: Sitagliptin, coronary ischemia/reperfusion, Bax, Bcl2 and cypD

ABBREVIATIONS

DPP-4 (dipeptidyl peptidase-4),I/R (ischemia/reperfusion), MI (myocardial infarction),p.o (oral), LDH (lactate dehydrogenase), CK-MB (creatine kinase-MB), cTn-I (cardiac troponin I), Bax (bcl2 associated X protein),cypD (cyclophilin D).

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INTRODUCTION

The detrimental effects of coronary artery diseases (CAD) are usually attributable to the effects of acute myocardial I/R injury. At the site of a vulnerable coronary plaque, platelets attach to the vessel wall and initiate thrombotic occlusion of the coronary vessel leading to myocardial ischemia and infarction [1].

Prompt reperfusion of ischemic myocardium is critical for restoring normal heart function. However, this return of blood flow can paradoxically enhance the destruction of reversibly damaged myocytes, thereby leading to the progression of myocardial dysfunction and infarction [2,3].

Cell death can be classified based on various criteria, but the major types are apoptosis, and necrosis [4]. Apoptosis was considered a regulated form of cell death (i.e. the cell starts its suicide program),necrosis is generally seen as a passive and unregulated process resulting from externally-induced cellular injury. Cell death following I/R has been reported to have features of both apoptosis and necrosis [5].

Necrosis was thought to be an unregulated process, but many studies suggest that necrosis can be regulated and that interventions can reduce necrotic cell death[5].This type of regulated necrotic cell death was later referred to as “necroptosis” [6]. It was suggested that a number of proteins in some way is involved in the process of necroptosis, one of special interest is cyclophilin D (CypD) [7].

DPP-4, also known as adenosine Deaminase complexing protein 2 or CD26, was first described in 1966 by Hopsu-Havu and Glenner by its enzymatic activity in rat liver [8,9]. The relevance of DPP-4 as a target in I/R has been demonstrated in several animal studies, mostly in MI [10,11]. Moreover, several DPP-4 substrates, account partly for the protective effects offered by the DPP-4 inhibitors. Also the release of several DPP-4 substrates are acutely regulated by ischemia and have beneficial effects in the I/R setting [12].

Several DPP-4 inhibitors like sitagliptin are currently available for the treatment of type 2 diabetes either as monotherapy or in combination with oral agents (metformin, glitazones, and/or sulphonylureas) and insulin [13].The euglycemic effects achieved by the use of DPP-4 inhibitors in diabetic patients account for the indirect positive effect DPP-4 substrates on cardiovascular diseases outcomeshowever, accumulating evidence from both experimental and clinical studies suggests direct pleiotropic actions of DPP-4 substrates on the cardiovascular system [14].

Based on these facts; the present study aimed to evaluate the cardioprotective effect of sitagliptin using an experimentally-induced coronary I/R mode in male rats as well as the possible interfering of sitagliptin with the apoptotic and/or necrotic pathways responsible for myocardial cell death.

MATERIALS AND METHODS

Animals:

Adult male Wistar rats (250–300 g) were purchased from National Research Center, Cairo, Egypt. Rats were kept under controlled conditions, with a 12 h light/dark cycles, at an ambient temperature of 22 ± 2°C and a humidity of 65–70%. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animals handling and experimental protocols were approved by the guidelines of the Research Ethical Committee of the Faculty of Pharmacy, Cairo University, Cairo, Egypt (Permit Number: PT 665). All the surgical processes were performed under urethane anesthesia and all efforts were made to minimize suffering.

Induction of coronary I/R:

Coronary I/R was induced in rats as previously described [15]. Briefly, rats were anesthetized with urethane (1.5 g/kg, I.M) [16], intubated, and ventilated with a rat ventilator (BioScience, Sheerness, Kent, UK). The depth of anesthesia was checked by toe pinch and the ECG leads were connected to record electrocardiographic changes during the I/R procedure (Power Lab, AD Instruments Pty Ltd., Australia). Left thoracotomy was performed *via* the third intercostal space, the lungs were retracted, the pericardium was opened and the left anterior descending (LAD) coronary artery was ligated 4 mm from its origin with a

slipknot using 6.0 Vilene suture (Dynek Pty Ltd, Australia). Myocardial blanching and electrocardiographic evidence of injury (elevation of ST segment height) confirmed ischemia; the ligature was released after 30 min. and reperfusion was confirmed visually.

Experimental design:

Rats were randomly subdivided into 3 groups (n = 18). Animals in the first and second groups served as the sham and I/R controls, respectively. Rats in the two groups received 1ml saline for 3 days.On the 4th day thoracotomy only was carried out for rats in the 1st group, while those in the 2nd group underwent I/R (30 min/2 hr) and ECG recording was performed. In group 3, rats were pretreated with sitagliptin (300mg/kg, p.o) for 3 days [17], then LAD coronary artery was subjected to I/R (30 min/2 hr) on the 4th day. Sitagliptin was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

At the end of the 2 hr reperfusion, hearts (n=6/group) were excised and the ventricles were used for staining, while the ventricles of the other twelve rats/group were separated, weighed and immersed immediately in liquid nitrogen and then stored at −80°C until analysis of the biochemical parameters.

Determination of infarction area:

As described before [18], ventricles which were assigned for staining were sliced transversely into 5-6 slices (5 mm thick) from apex to the occlusion site. Slices were incubated for 10 min in 1% 2,3,5-triphenyl-tetrazolium-chloride (TTC) (Bio Basic Inc., Ontario, Canada) buffer solution (pH 7.4) at 37°C to stain the viable tissue brick red, while the infarcted areas were left unstained (pale). Each slice was photographed using a digital camera (Nikon, COOLPIX, 16.0 Megapixels) and the pale infarcted area was determined planimetrically using Image software (version 1.48g), and the area of infarction was calculated as a percentage of the whole slice area.

Determination of ST-segment height elevation:

After a stabilization period, ECG was recorded for each rat, and the height of the ST segment was recorded as a baseline, just before LAD coronary artery ligation. After I/R (30 min/2 hr), the ECG analysis module (Power Lab, AD Instruments Pty Ltd., Australia) was used to calculate the ST segment elevation from baseline for each rat.

Ventricular tissue preparation:

The frozen ventricles (n=12/group) were used for the assessment of cardiac enzymes and the mRNA expression levels of apoptotic and necrotic markers.

For the biochemical analysis, ventricular tissues of 6 rats/group were homogenized in phosphate buffer saline (Lonza, Verviers, Belgium) and the homogenates were centrifuged at 5000 x g 8°C for 5 min. Afterwards the supernatant was then divided into several aliquots for measuring the content/activity of cardiac enzymes.

For the detection of mRNA gene expression of apoptotic and necrotic markers, the other six ventricles/group were disruptedin a lysis buffer containing guanidine is othiocyanate and homogenized for total mRNA extraction.

Assessment of cardiac enzymes:

Cardiac lactate Dehydrogenase (LDH) activity was measured in tissue homogenates using a test reagent kit (Biosystems, Barcelona, Spain) and creatine kinase-MB (CK-MB) was assayed quantitatively using a biochemical kit (Anti CK-M. Immunoinhibition, Kinetic UV. Liquid, Spinreact, Vall de Bas, Spain)(Rat Creatine Kinase MB Isoenzyme (CK-MB) ELISA, Seattle WA, USA) according to the method described by the manufacturer. Cardiac troponin I (cTn-I) concentration in tissue homogenates was done using specific ELISA kit (CUSABIO,Wuhan, China) according to the manufacturer's prescripts.

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Detection of Bax, Bcl2 and Cyp D gene expression using real time PCR (RT–PCR) :

RNA extraction and cDNA synthesis:

Total RNA was isolated from ventricular tissue homogenates using RNeasy Purification Reagent (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The purity (*A*260/*A*280 ratio) and the concentration of RNA were obtained using spectrophotometry (Gene Quant 1300, Uppsala, Sweden). RNA quality was confirmed by gel electrophoresis and 4 µg of total RNA was used to synthesize the first-strand cDNA using an Oligo-dT primers and Superscript™ II RNase Reverse Transcriptase; this mixture was incubated at 42°C for 1h. The kit was supplied from Super Script Choice System (Life Technologies, Breda, Netherlands).

Real-time quantitative polymerase chain reaction (RT-PCR):

RT-PCR amplification was carried out as previously described [19] by using 10 µL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Bio systems, CA, USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers; the sequences of PCR primer pairs used for each gene are shown in Table 1. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Bio systems, CA, USA). PCR reactions were done in 95°C for 10 min(1 cycle), 94°C for 15 s, and 60°C for 1 min (40 cycles). Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1·7 sequence detection software (PE Bio systems, CA, USA). Relative expression of the studied genes was calculated using the comparative threshold cycle method. All values were normalized to the GAPDH gene.

Statistical Analysis:

Data are reported as means ± S.E.M. Statistical differences were executed using one-way analysis of variance (ANOVA), followed by Tukey's*post hoc* test or student t-test. The differences were considered to be significant at $P<0.05$.

RESULTS

Effects of different treatments on infarct size:

Rats subjected to LAD coronary artery ligation for 30 min followed by 2 hr reperfusion (30 min/2h; I/R) showed an increase ininfarct size (MI; 61.6%). Pretreatment with sitagliptin, however, caused 62% reduction in the infarct size as compared to the I/R insult (Fig. 1).

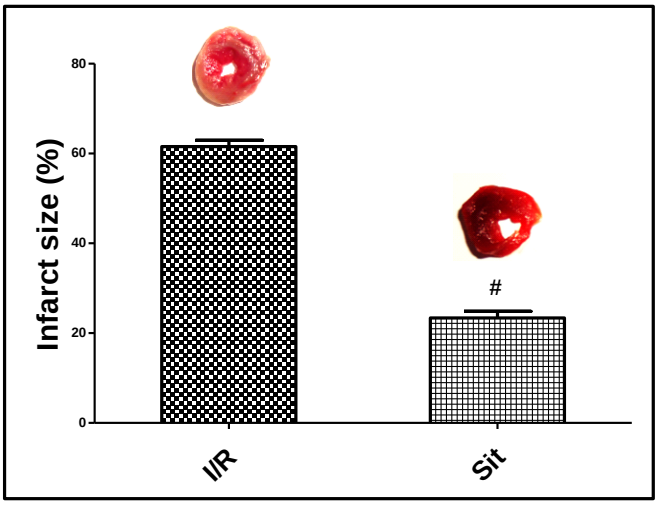

Fig 1: Effect of I/R (30 min./2h) and sitagliptin (Sit; 300 mg/kg, p.o) on infarct size. TTC staining of rat ventricles showing pale infarcted areas. I/R and Sit groups received either saline or Sit for 3 days then LAD coronary artery was subjected to I/R (30 min./2h) on the 4th day. Values are means of 6 rats ± SEM.As compared to I/R (#) (Student t-test;*P*< 0.05).

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Effects of different treatments on ST segment height:

Fig. (2 & 3) depicted that I/R (30 min/2h) elevated ST segment height to 0.16 mV from base line, while pre-administration of sitagliptin markedly suppressed the elevated ST segment by 72.6% as compared to I/R group.

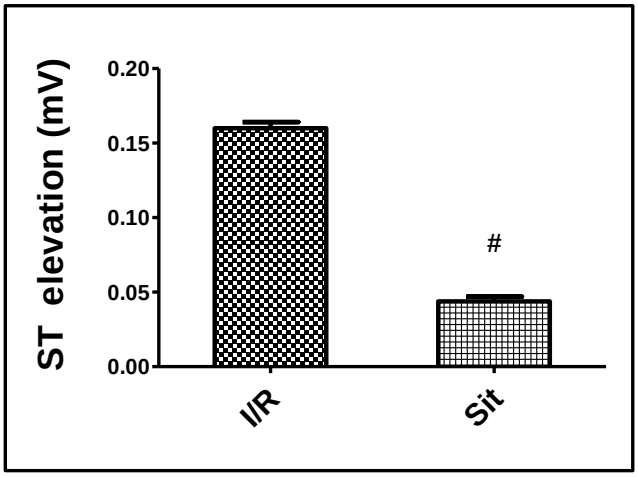

Fig 2: Effect of I/R (30 min./2h) and sitagliptin (Sit; 300 mg/kg, p.o) on ST segment elevation (mV). I/R and Sit groups received either saline or Sit for 3 days then LAD coronary artery was subjected to I/R (30 min./2h) on the 4th day.. Values are means of 12 rats ± SEM.As compared to I/R (#) (student t-test; *P*< 0.05).

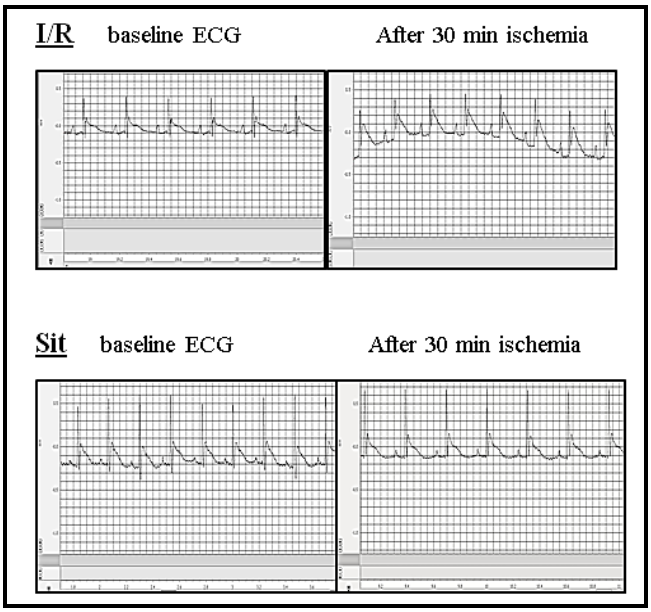

Fig 3: ECG changes (ST segment elevation) following I/R (30 min./2h) and sitagliptin (Sit; 300 mg/kg, p.o). I/R and Sit groups received either saline or Sit for 3 days then LAD coronary artery was subjected to I/R (30 min./2h) on the 4th day.. Values are means of 12 rats ± SEM.As compared to I/R (#) (student t-test; *P*< 0.05).

Effects of different treatments on cardiac enzymes (LDH, CPK-MB and cTn-I):

Fig. 4 demonstrated thatI/R (30 min/2 hr) increased cardiac activity of LDH (1.71 fold), and boosted the contents of CPK-MB (4.63 folds) and cTn-I (8.7 folds), as compared to the sham-operated group. Sitagliptin pretreatment for 3 days before induction of LAD coronary artery I/R markedly reduced the elevated cardiac enzyme by 36.62, 62.93 and 74.78 %, respectively, as compared to the I/R group..

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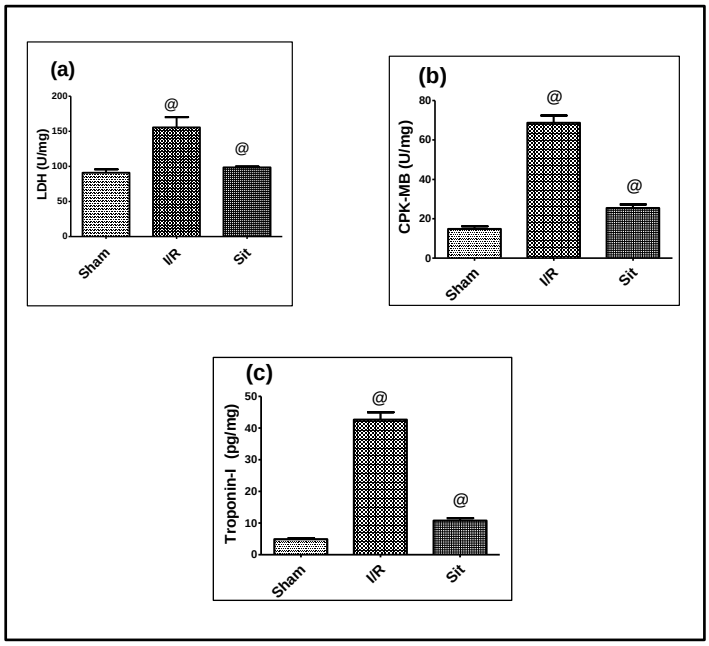

Fig 4: Effect of I/R (30 min./2h) and sitagliptin (Sit; 300 mg/kg, p.o), on cardiac levels of (a) lactate dehydrogenase (LDH), (b) creatine phosphokinase (CPK-MB) and (c) troponin-I. Sham, I/R and Sit groups received saline or Sit for 3 days then LAD coronary artery was subjected to I/R (30 min./2h) on the 4th day in all groups, except the sham one. Values are means of 6 rats ± SEM.As compared to sham ($^{@}$) and I/R ($^{\#}$) (one –way ANOVA followed by Tukey's post hoc test); *P*< 0.05.

Effects of different treatments on mRNA expression levels of Bax, Bcl2 &Cyp D:

On the molecular level, Fig. 5 showed that the induction of MI in the I/R group noticeably increased the mRNA expression levels of Bax and Cyp D by 10.5 and 11.18 folds, respectively, and depleted that of the anti-apoptotic marker Bcl2 (90.3%), as compared to the sham control group. Pretreatment with sitagliptin downregulated the mRNA expression levels of the apoptotic (Bax, 76.7%) and necrotic (Cyp D, 81%) markers, and elevated the antiapoptotic marker Bcl2 (3.7 folds), as compared to the I/R group.

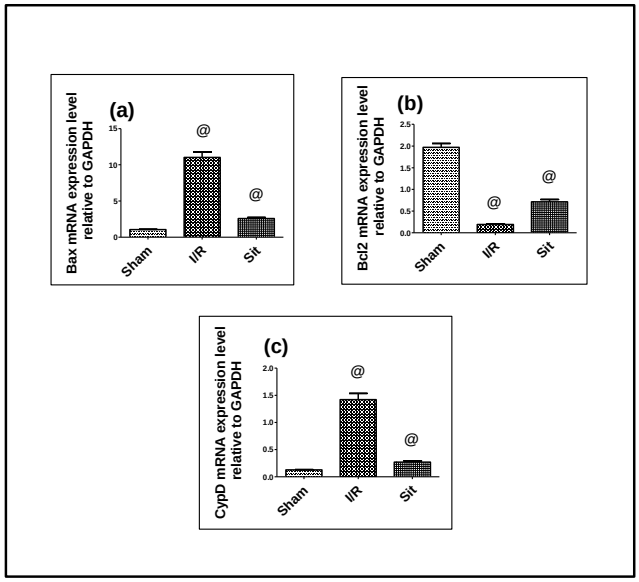

Fig 5: Effect of I/R (30 min./2h) and sitagliptin (Sit; 300 mg/kg, p.o) on mRNA expression levels of (a) Bax, (b) Bcl2 and (C) CypD. Sham, I/R and Sit groups received saline or Sit for 3 days then LAD coronary artery was subjected to I/R (30 min./2h) on the 4th day in all groups, except the sham one. Values are means of 6 rats ± SEM.As compared to sham ($^{@}$) and I/R ($^{\#}$) (one –way ANOVA followed by Tukey's post hoc test); *P*< 0.05.

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DISCUSSION

Ischemic heart diseases and stroke are the leading causes of death worldwide [20]. Since 1990, more people have died from CAD than any other death cause [21]. MI is the serious and often fatal consequence of CAD which results from the acute occlusion of one of the major coronary arteries leading to myocardial ischemia; a diminished blood supply to the heart [22]. Thus, the continuous searching for novel cardioprotective strategies has been a matter of great interest.

In the present work, LAD coronary artery ligation for 30 min followed by 2 hr reperfusion induced myocardial ischemia and a subsequent MI in the rats of the I/R group as compared to those in the sham group.The importance of the duration of the ischemic insult in determining the outcome of an episode of ischemia followed by reperfusion has been studied previously [23]. In the current work, 30 min coronary ischemia followed by 2 hr reperfusion succeeded to induce typical MI which was confirmed by the significant increase in the infarct size, obvious ST segment height elevation and increased cardiac enzymes levels as compared to the sham group. These events that occur subsequent to coronary artery occlusion are termed myocardial I/R denoting the distinct phases of cellular injury with ATP depletion, lactate accumulation and acidosis observed during ischemia and the production of reactive oxygen and nitrogen species during reperfusion as well as activating several protein kinase pathways [24].

Similarly, previous studies proved that when the duration of the ischemic episode was extended past 15 and up to 60 minutes myocardial “irreversible injury” occurred. This injury was associated with cell death, since restoration of blood flow did not result in salvage of the tissue but rather accelerated cellular disintegration and irreversible changes in ultrastructure [25,26]**.** This is because restoration of arterial blood flow was associated with a series of catastrophic changes in the ultrastructure of subendocardial myocytes that were documented by electron microscopy such as marked cellular swelling, myofilamenthypercontracture, large areas of membrane disruption, and calcification of the mitochondria [26].

In the current study, pretreatment with sitagliptin obviously protected against the coronary I/R insult, as it decreased infarct size, ST segment height and the elevated cardiac enzymes. These findings coincide with previous studies using sitagliptin [3,17], vildagliptin [27]and linagliptin [15].

It is well known that two morphologically distinct pathways contribute to myocytes cell death after myocardial I/R injury; *viz*., necrosis and apoptosis, and that myocardial injury results from a significant increase in both events as documented previously [7,28] and herein. Induction of myocardial I/R overexpressed the pro-apoptotic marker Bax, while decreasing the anti-apoptotic Bcl2, as compared to the sham control. Earlier studies also demonstrated that I/R is a stronger stimulus of acute apoptosis than permanent occlusion [29] and that overexpression of Bcl2 and Bax ablation protect against I/R [30].

On the other hand, induction of coronary I/R, in our study, over expressed Cyp D as compared to the sham control. Cyp D is known as a key regulator of mitochondrial permeability transition pores (mPTP) and necrotic, but not apoptotic, cell death [31] and that deletion of ppif, the gene encoding for Cyp D, prevented mPTP opening, while its overexpression induced strongly mPTP opening leading to necrotic cell death [32]. Thus, our agreement with these earlier studies confirms the contribution of both apoptotic and necrotic pathways in the I/R- induced cell injury.

However, pretreatment with sitagliptin decreased markedly the elevated expression levels of Bax and Cyp D, while increased that of Bcl2, as compared to I/R group. Sitagliptin-mediated cytoprotective properties were confirmed previously in different models in kidney [33] and pancreas [34]. These effects can correlate with the sitagliptin-mediated decrease in infarct size, proven herein.

Accordingly, the results of the present study confirms the cardioprotective effect of sitagliptin which is mediated partly through the its anti -apoptotic and -necrotic actions that played a role in decreasing the I/R-induced cell injury. Thus, targeting the previous pathway could help in finding new cardioprotective agents.

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