

Possible Protective Mechanisms of Sitagliptin against Isoproterenol Induced Myocardial Injury in Rat

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Abstract Sitagliptin, dipeptidyl peptidase 4 inhibitors, has been investigated and proved to improve triglycerides, low density lipoprotein and blood pressure, which are important risk factors for cardiovascular diseases. **Aim:** The present study was designed to investigate the potential protective effect of sitagliptin in isoproterenol induced myocardial injury. Further assessment was done to address the cardioprotective mechanism. **Method:** Male Wister rats were treated with sitagliptin (30 mg/kg/day for 2 weeks by gavage) and/ or isoproterenol (85mg /kg; i.p. 24 hours apart at day 14th and 15th of the experiment). **Results:** Isoproterenol induced a significant ECG changes, several pathological changes and elevated cardiac enzymes. These changes were significantly attenuated by pre-treatment of rats with sitagliptin. As a marker of oxidative stress, isoproterenol caused significant decrease in reduced glutathione level and superoxide dismutase with increase in malondialdehyde compared to the control group. Sitagliptin pretreatment restored these markers toward normal values. Energy decline was assessed by measuring ATP/ADP, which decreased significantly in isoproterenol group and significantly increased by sitagliptin pretreatment. Isoproterenol caused inflammatory effects indicated by up-regulation of tumor necrosis factor- α expression in the myocardial tissue. Sitagliptin also counteracted inflammatory cell infiltration, other histopathological changes, and the overexpression tumor necrosis factor- α in myocardial tissue. Collectively, these findings suggest that sitagliptin, an anti-diabetic agent, has cardioprotective effect against isoproterenol induced myocardial injury that could be through antioxidant properties, TNF- α inhibition, and an enhancement of myocardial energy state.

Keywords Isoproterenol (ISO); Sitagliptin (SL); ATP/ADP, oxidative stress; myocardium

Introduction

The cardiac problems in patients with diabetes is a major cause of morbidity and mortality (Laing et al., 2003; Snell-Bergeon and Wadwa, 2012), it affects 50-60% of persons with type 2 diabetes (Duckworth et al., 2009). Diastolic dysfunction is the common effect of type 2 diabetes on cardiovascular system (Patil et al., 2011). Insulin resistance is associated with chronic low-grade inflammation, which is predisposing to hypertension and chronic heart failure (Shim et al., 2006; El-Bassossy et al., 2015). So an anti-diabetic agent that could improve cardiovascular outcomes in these patients is essentially to be known.

Isoproterenol (ISO), a β -receptor agonist, administration caused significant left ventricular hypertrophy and fibrosis, and decreased left ventricular diastolic function. ISO-treated rats are an appropriate model of diastolic dysfunction (Sumita Yoshikawa et al., 2012). It depletes the energy source of myocytes, which results in irreversible cellular damage and

ultimately infarct-like necrosis (Murugesan and Manju, 2013).

Glucagon-like peptide-1 (GLP-1) is secreted primarily by the intestinal entero-endocrine cells (Nauck et al., 1993). GLP-1 analog showed protective effects on high-fat diet-induced insulin resistance, inflammation (Apaijai et al., 2012), and myocardial infarction (Arakawa, et al., 2010). It improve energy metabolism by the intake of glucose to myocytes (Barakat et al., 2011). GLP-1 also activates c-AMP-dependent protein kinase, thus enhancing L-type Ca^{2+} current in isolated cardiomyocytes (Nikolaidis et al., 2004; Anagnostis et al., 2011). It is degraded by dipeptidyl peptidase-4 (DPP4) with a very short half-life (2 to 3 min) (Xiao et al., 2011). Recent experimental studies showed the protective effect of DPP4 inhibitors in hypertension (Pacheco et al., 2011), heart failure (Chaykovska et al., 2011), and myocardial infarction (Miki et al. 2012).

Sitagliptin (SL) was the first DPP-4 inhibitor marketed in the United States in 2007. Vildagliptin, was the second approved in Europe and other countries, followed by sitagliptin in 2009 (Miki et al., 2012). DPP-4 inhibitors have been investigated and proved to improve triglycerides, low density lipoprotein cholesterol, high density lipoprotein cholesterol, and blood pressure, which are important risk factors for cardiovascular diseases (Drucker et al., 2010). SL specifically has been approved by the FDA. It can be used as a single therapy or combined with metformin or glitazone to treat type 2 diabetes (Aschner et al., 2006; Charbonnel et al., 2006).

Therefore, the goal of the present study was designed to investigate the possible protective effects of SL against ISO-induced myocardial toxicity in rat. Moreover, to explore the role of oxidative stress, tumor necrosis factor (TNF)- α , as well as the energy decline as possible mechanisms.

Material and method

Drugs and chemicals

Isoproterenol (ISO) was purchased as powder from Sigma Chemicals (USA). Isoproterenol was dissolved in saline. Sitagliptin (Januvia® tablet) was obtained from Merck Sharp & Dohme Ltd.

Animals

Thirty-two Male Wistar rats weighed 200-220g were obtained from the Holding Company for Biological Products & Vaccines VACCERA, Egypt. The chow was acquired from Meladco for Animal Food, Egypt. Pellets and tap water were provided ad libitum. Temperature was maintained at 25°C. A 12/12 h light/dark cycle was maintained. Rats were allowed at least 1 week to acclimatize to the lab conditions. All procedures were done according to European Directive 2010/63/EU and guidelines of ethical Committee, faculty of Medicine, Ain Shams University.

Study Design

Rats were allocated into four groups (n=8) and treated for 15 days: vehicle control group, SL group (oral Sitagliptin 30 mg/kg/day), ISO group (intraperitoneal isoproterenol 85 mg/kg/day, twice at an interval 24 hours on the 14th and 15th day) (Murugesan and Manju, 2013) and ISO-SL group. SL concentrations were selected based on a previous report which showed its effectiveness in cardio-protection in obese insulin resistant rats (Chen et al., 2011; Apaijai et al., 2013).

Twenty-four hours after last injection with ISO, assessment of electrocardiograph under urethane anesthesia (1.2g/kg; Sigma -Aldrich) was done. After blood sampling from the retro-orbital venous plexus, it is allowed to clot and serum was separated by centrifugation at 3000 g for 15 min for biochemical assessment. Then rats were killed. The heart were dissected out, weighed and immersed in ice-cold saline or fixed in 8% buffered formalin for histopathological examination.

ECG assessment

By using ECG apparatus CardiMax FX-7102, USA, Electrocardiography (ECG) was recorded 24h after

ISO injection. RR interval, heart rate and ST segment deviations were measured. ECG recording speed was 25 mm/second.

Cardiotoxicity markers

Heart index was calculated according to the formula: (heart weight/body weight) \times 100 Creatine kinase-MB and troponine I were determined using an enzyme-linked immunosorbent assay (ELISA) developed by Life Diagnostics Inc. West Chester PA, USA, according to the manufacturer's instructions.

Assessment of oxidative stress markers

To assess the oxidative stress, the tissue level of, SOD, GSH and MDA were measured in cardiac homogenate of the different treated groups. SOD activities in the cardiac samples were assessed using assay kits (Trevigen, Inc., USA) according to the manufacturer's instructions. GSH was assessed according to the method of Ellman (Ellman, 1959). Also, MDA was measured according to the method of Mihara and Uchiyama (Mihara and Uchiyama, 1978).

Assessment of myocardial nucleotides

Hearts were homogenized in 6% perchloric acid. The clear supernatant was then neutralized by potassium hydroxide and used for determination of myocardial ATP and ADP by using HPLC (Kontron, 322, Sebai, Italy), C18 hypersil column and UV detector at 254 nm (Neri et al., 1986).

Assessment of myocardial TNF- α

Myocardial TNF- α was assessed immunohistochemical. Paraffin embedded heart tissue sections of 3 micron thickness were prepared. The sections were stained with ready to use primary antibody (rabbit polyclonal antibody to rat TNF- α (US Biological, USA). Hematoxylin was used as a counter staining and the slides were visualized under light microscope. The quantification of staining was performed using image analysis software (Image J, 1.46a, NIH, USA).

Histopathological assessment

Autopsy samples from all groups were taken from heart (mainly ventricle) and fixed in 10 % formalin for 4-7 days, followed by dehydration, clearing and embedding in paraffin. Sections were cut 4-6 μ m and stained with H&E.

Statistical analysis

Values were expressed as mean \pm SD. One-way ANOVA followed by Tukey's test Multiple Comparison Test were used to assess differences between groups. Categorical data was compared using a chi-square test. All statistical analysis was done using GraphPad Prism (GraphPad Prism software, version 5). The differences were considered significant when the calculated $P < 0.05$. Correlation analyses were performed with the help of the Pearson's correlation coefficients.

Results

ECG

ECG tracing showed normal cardiac activity in the control and SL per se treated rats. Rats in ISO-treated

group showed significant increase in the heart rate by 28% compared to the control group with significantly increased elevated ST segment frequency from 12.5% in the control to 87.5% in ISO group. Such ECG abnormalities were obviously statistically improved in ISO intoxicated animals pretreated with sitagliptin as evidenced by decreased HR by 22.7% as compared to ISO group and decreased the incidence of elevated ST segment within group to be 25% in ISO+ SL group (Figure 1).

Cardiotoxicity markers

Rats treated with ISO showed a significant increase in the relative heart weight by 21.2% compared to the control group. On the other hand, pretreatment with SL decreased it by 20% as compared to ISO animals. SL alone did not modify the absolute and relative heart weights (Table 1). The activities of myocardial injury markers, CK-MB and troponin I, compared to the control group were significantly elevated in the ISO animals by 155 and 156% respectively. Pretreatment with SL in ISO intoxicated animals significantly reduced the activities of CK-MB and troponin I as compared to ISO animals. SL alone, when compared to the control group, did not show any significant changes in CK-MB and troponin I (Table 1).

Assessment of oxidative stress markers

As shown in Table 2, ISO treatment significantly reduced GSH level by 68.8% and increased MDA level by 181.7% as compared to the control group. Furthermore, ISO induced a significant decrease in the cardiac antioxidant enzyme activities SOD by 40.4% as compared to the control levels. Pretreatment with SL in ISO animals could significantly elevate the levels of both of GSH and SOD by 83.2% and 35% respectively and reduce the MDA levels by 40.5% as compared to ISO animals (table 2).

Assessment of myocardial nucleotides

Rats treated with isoproterenol as compared to the control values showed a significant decrease in myocardial ATP and ADP by 59.4% and 36.1% respectively, as well as significant decrease in ATP/ADP ratio by 37.2% (Figure 2, table 3). SL pretreatment in ISO animals counteracted the decrease in ATP/ADP ratio induced by ISO approximately by 58.1% as compared with the ISO value (Figure 2). A significant negative correlation between ATP/ADP ratio and serum CK-MB ($r=-0.49$, $p<0.05$) (figure 2C).

Anti-TNF- α effect

The expression of myocardial TNF- α was estimated using immunohistochemical staining. Control group showed minimal immunostaining. ISO elevated the myocardial expression of TNF- α as shown by the intense brown staining while pretreatment of intoxicated animals with sitagliptin prevented this elevation to a large extent. No changes in myocardial expression were observed in the group treated with sitagliptin alone (figure 3A, 3B, 3C, 3D). The immunohistochemical staining was quantified as optical density (OD) of the stained regions using the image analysis software (figure 3E).

Histopathological assessment

To further characterize the cardiotoxicity induced by ISO, histopathological examination of heart tissues were done. Myocardial tissues from control and SL only treated rats showed normal architecture (Figure 4A, B). Histological examination of hearts from ISO-intoxicated animals revealed marked myocardial degeneration in the form of myofibrillar loss, cytoplasmic vacuolization, inflammatory cell infiltration, and congestion (Figure 4C). Pretreatment of intoxicated animals with SL preserved the normal myocardium architecture with remaining some inflammatory cell infiltrations (Figure 4D).

Table (1): Effect of sitagliptin (30mg/kg/day) on parameters of cardiotoxicity in isoproterenol induced myocardial injury

Groups	Heart index	CK-MB (IU/L)	Troponine I (ng/ml)
Control	0.33±0.03	89.83±8.6	1.083±0.29
sitagliptin	0.33±0.04 ^b	95.5±6.2 ^b	1.117±0.37 ^b
ISO	0.40±0.02 ^a	229.2±39.3 ^a	2.783±0.58 ^a
ISO+seaglebtin	0.32±0.04 ^b	140±29.9 ^b	1.443±0.35 ^b

Data are expressed as mean \pm SD ($n=8$). ISO isoproterenol, CK-MB creatine kinase; a or b significantly different from the normal control or ISO injected rats respectively at $P<0.05$ using ANOVA followed by Tukey–Kramer as a post hoc test.

Table (2): Effect of sitagliptin (30mg/kg/day) on oxidative stress in isoproterenol induced myocardial injury

Parameters	MDA (nmol/g tissue)	GSH (μ mol/g tissue)	SOD (μ g/g tissue)
Control	20.25±4.9	0.635±0.17	23.85±2.8
sitagliptin	19.97±4.4 ^b	0.5367±0.05 ^b	24.75±2.8 ^b
ISO	57.05±5.6 ^a	0.1983±0.05 ^a	14.22±3.6 ^a
ISO + sitagliptin	33.97±5.4 ^b	0.3633±0.04 ^b	19.2±2.6 ^b

Data are expressed as mean \pm SD ($n=8$). ISO isoproterenol, SOD Superoxide dismutase, GSH Reduced glutathione, MDA Malondialdehyde; a or b significantly different from the normal control or ISO injected rats respectively at $P<0.05$ using ANOVA followed by Tukey–Kramer as a post hoc test.

Table (3): Effect of sitagliptin (30mg/kg/day) on ATP, ADP, ATP/ADP in isoproterenol induced myocardial injury

Groups	ATP($\mu\text{mol/g}$ tissue)	ADP($\mu\text{mol/g}$ tissue)	ATP/ADP
Control	0.35 \pm 0.05	1.67 \pm 0.21	0.216 \pm 0.05
sitagliptin	0.35 \pm 0.05 ^b	1.73 \pm 0.16 ^b	0.2 \pm 0.04 ^b
ISO	0.14 \pm 0.03 ^a	1.065 \pm 0.122 ^a	0.136 \pm 0.03 ^a
ISO+seaglebtin	0.29 \pm 0.049 ^b	1.3 \pm 0.151 ^b	0.22 \pm 0.03 ^b

Data are expressed as mean \pm SD (n=8). ISO isoproterenol, CK-MB creatine kinase; a or b significantly different from the normal control or ISO injected rats respectively at $P < 0.05$ using ANOVA followed by Tukey–Kramer as a post hoc test.

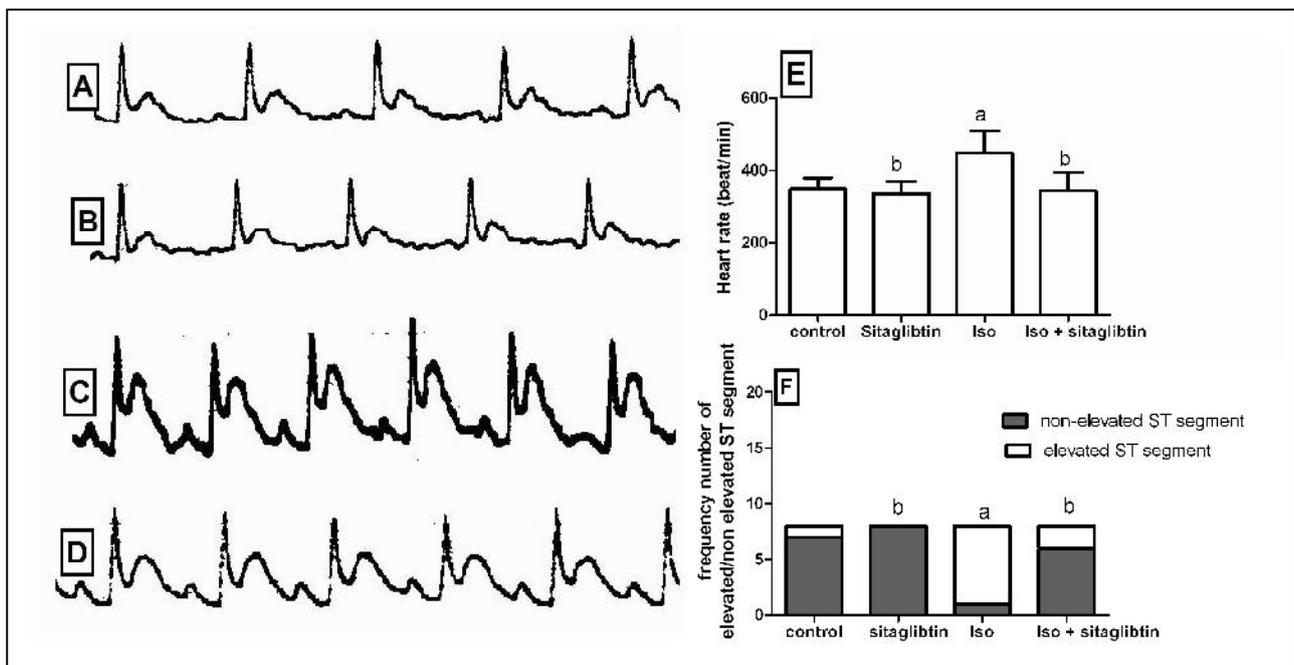


Figure (1): Effect of sitagliptin pretreatment on ISO-induced myocardial injury in ECG pattern. (A) Control group, (B) sitagliptin (30 mg/kg) treated group, (C) ISO (85 mg/kg) treated group, (D) sitagliptin (30 mg/kg). ECG tracings of control and sitagliptin-only treated rats show normal pattern. ISO-treated group shows tachycardia, elevation in ST segment. Sitagliptin pretreatment in ISO intoxicated rats significantly decrease heart rate, ST segment elevation. (E) Heart rate data are expressed as mean \pm SD (n=8). (F) Frequency of elevated to non-elevated ST segment. a or b means significantly different from the normal control or ISO injected rats respectively at $P < 0.05$ using ANOVA followed by Tukey–Kramer as a post hoc test. For frequency data a chi-square test was used.

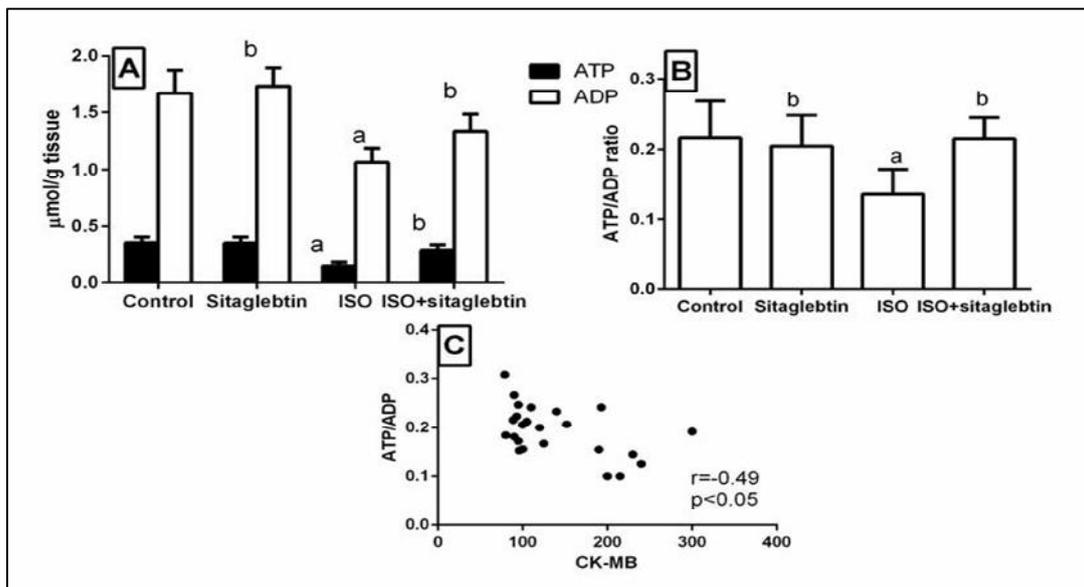


Figure (2): Effect of sitagliptin pretreatment on ISO-induced myocardial injury (A) myocardial nucleotide ATP and ADP and (B) ATP/ADP ratio. (C) A correlation between serum cardiac enzyme CK-MB and ATP/ADP ratio. $n=8$, a and b significantly different from the normal control or ISO injected rats respectively at $P<0.05$ using ANOVA followed by Tukey–Kramer as a post hoc test. Correlation analyses were performed with the help of the Pearson’s correlation coefficients

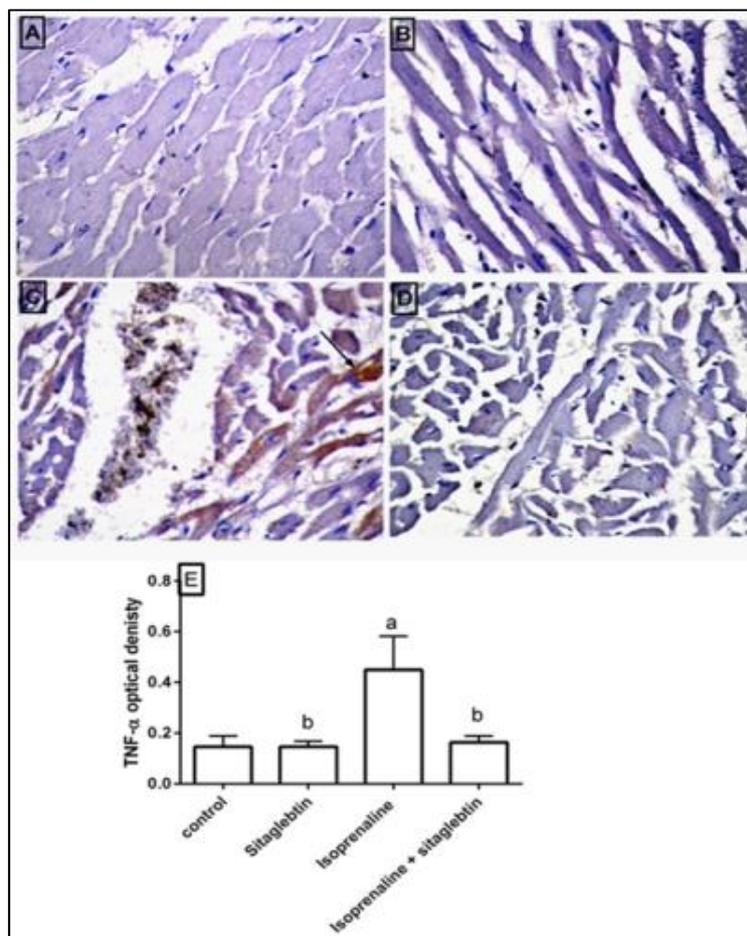


Figure (3): Effect of sitagliptin pretreatment in isoproterenol induced myocardial injury on tissue TNF- α expression by immunohistochemical analysis of heart tissue (200 \times). (A) control group, (B) sitaglebtin (30mg/kg) treated group, (c) ISO intoxicated rats shows more immunostaining (arrow; brown colors), (d) sitaglebtin pretreatment in ISO intoxicated rats showed less staining, (E) graphic representation of optical density (mean \pm SD determined by direct visual counting of ten fields for each of three slides. $n=8$, a and b significantly different from the normal control or ISO injected rats respectively at $P<0.05$ using ANOVA followed by Tukey–Kramer as a post hoc test.

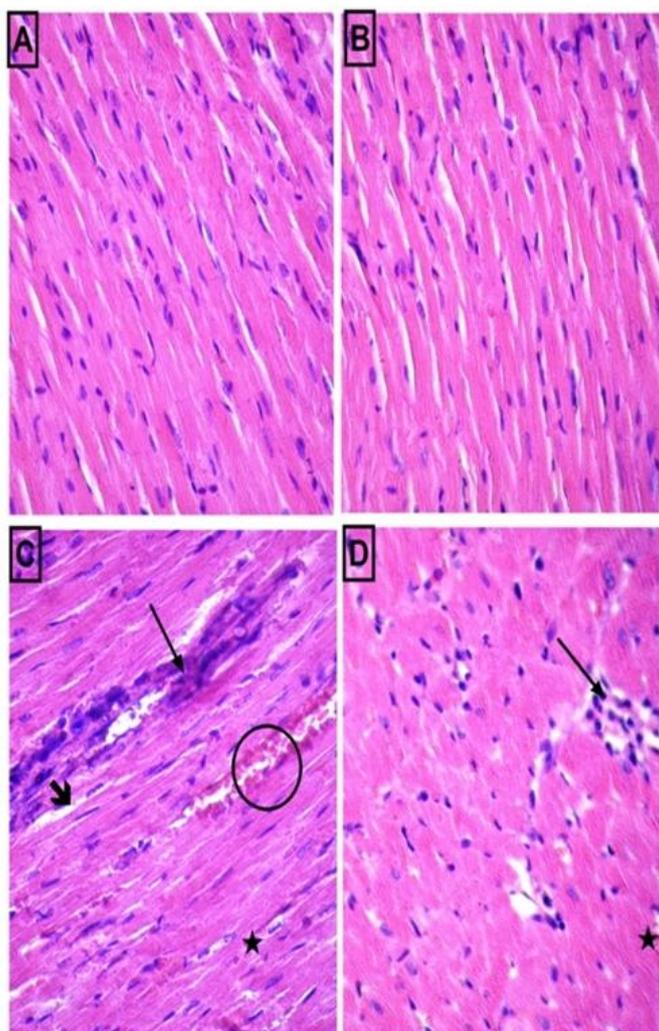


Figure (4): Effect of sitagliptin pretreatment on ISO-induced histological alterations of the heart tissue (200×). Photomicrographs of haematoxylin and eosin stained sections of heart depicting (A) control group, (B) sitagliptin (30 mg/kg) treated group, (C) ISO treated group, (D) sitagliptin (30 mg/kg) pretreatment in ISO rats. (A) and (B) show normal histoarchitecture of the rat heart. (C) shows ISO-induced dilated congested blood vessels (circle), cytoplasmic vacuolization (stars) and inflammatory cell in filtration (long arrows). Widely separated muscle fibers leaving empty spaces between them (short arrow). (D) Shows that sitagliptin pretreatment prevented cardiomyocyte damage induced by ISO where there was still some inflammatory cell in filtration (long arrows).

Discussion

DPP-4 inhibitors are newly available drugs approved for the treatment of type 2 DM mainly by improving meal-stimulated insulin secretion by pancreatic β -cells, which is accomplished by sparing the hormone glucagon-like peptide-1 from degradation by the enzyme DPP-4 (Ahren et al., 2000). Glucagon-like peptide-1 receptors have been reported to be widely expressed in the heart and vasculature with specific localization in vascular endothelium/smooth muscle, endocardium and cardiomyocytes, suggesting that GLP-1 may play an important role in the cardiovascular system (Forst et al., 2012). Experimental data from animal and human studies indicate that GLP-1 has inotropic and vasodilatory effects, increased myocardial glucose uptake, improvement of endothelial function, reduction in infarct size, as well as potential anti-inflammatory and antiatherogenic actions (Goyal et al., 2010). Recent

work has indicated that DPP4 inhibitor or glucagon-like peptide-1 (GLP-1) receptor agonists exert cardiovascular effects, such as modulation of heart rate, blood pressure, vascular tone and myocardial contractility (Okerson et al., 2014; Gill et al., 2010)

The present study was conducted to evaluate the possible protective effects of sitagliptin (SL) against ISO-induced myocardial toxicity in rat. Moreover, to explore the possible mechanisms. ISO-induced myocardial injury was assessed by ECG, cardiac enzymes and histopathological examination of heart tissue. Significant alterations of ECG patterns were observed in ISO-intoxicated rats when compared with normal rats. The characteristic findings were the elevation of the ST segment, which might be an indicative sign of ischemia. ISO also increased the heart rate. Pretreatment with SL showed a protective effect against ISO-induced altered ECG patterns. This

is Consistent with previous reports done by Apaijai and others who demonstrated that both vildagliptin and sitagliptin share similar efficacy in cardioprotection in obese insulin-resistant rats (Apaijai et al., 2013).

In addition, cardiac enzymes CK-MB and troponin I were increased with several histopathological changes in ISO intoxicated rats, which are in accordance with previous studies (Murray et al., 2000). Cardiac troponin I has been shown to be specific and sensitive biomarker of drug-induced myocardial cell injury in animals (Wallace et al., 2004). In rats, a number of studies have described a relationship between the serum levels of cardiac troponin I and the severity of ISO-induced myocardial injury (Bertinchant et al. 2000). When myocardial cells, containing cardiac enzymes are damaged due to ischemia, the cell membrane becomes permeable, which results in the leakage of enzymes. This accounts for the increased activities of these enzymes in serum. In patients, troponin provides an information about the severity of myocardial ischemia (Van Der Laarse, 2002). ISO-induced myocardial alterations are similar in certain respects to those occurring in human beings following a myocardial infarction (Zhang et al., 2008). In the present study, SL pretreatment prevented the changes in heart index and cardiac enzymes induced by ISO, indicating its beneficial cardioprotective effect. In a previous pilot study showed that the addition of dipeptidyl peptidase-4 inhibitor therapy with sitagliptin to the treatment regime of patients with type 2 diabetes mellitus and coronary artery disease is associated with a sustained improvement in myocardial performance during dobutamine stress and a reduction in postischemic stunning (McCormick et al., 2014).

It has been previously reported that, caecholamines produce quinones with subsequent myocardial Oxidative damage. So oxidative stress and apoptosis may contribute in the pathogenesis of these changes (Mann, 1998; Remiao et al., 2001). It is thought that the β adrenergic cardiac stimulating activity exerted by ISO increases cardiac oxidative metabolism to a level that exceeds the amount of oxygen available to the myocyte through the unobstructed coronary circulation leading to energy imbalance (Van Vleet et al. 2002). The left ventricular subendocardium is the most susceptible area to hypoxia caused by tachycardia (Van Vleet et al., 2002).

In the present work, ISO intoxicated rats showed a significant increase in myocardial lipid peroxides and significant decreases in GSH and SOD activities as compared with the control group. Pretreatment with SL in ISO intoxicated animals ameliorated these changes. Ferreira and others showed a remarkable positive impact of chronic sitaglebtin treatment at dose of 10mg/kg/day on lipid peroxidation in both the pancreas and the heart in animal model of type 2 DM (Ferreira et al., 2010). This might be a further advantage in the management of diabetes and its proatherogenic comorbidities.

A state of energy starvation was observed in the present study as proved by a significant decrease in myocardial ATP, ADP and ATP/ADP ratio as compared to the control, this was in accordance with previous report by Chagoya de Sanchez et al (Chagoya de Sanchez et al., 1997). SL induced a significant increase in ATP/ADP ratio as compared with ISO group. Furthermore, a significant negative correlation between ATP/ADP ratio and CK-MB. Thus not only the antioxidant properties of SL could explain the cardioprotective effect but also its enhancement effect on the cellular energy. A constant re-synthesis of ATP by oxidative phosphorylation in the mitochondria is essentially needed to maintain proper function and calcium utilization by myocytes (Zhang, 2002).

Inflammation is a key pathophysiologic factor in diabetic cardiomyopathy (Cas et al., 2013). Biomarkers of inflammation including TNF- α levels is associated with risk of developing type 2 diabetes (Ti et al., 2011). ISO, was known to induce expression the myocardial inflammatory mediators as TNF- α , IL-6, and IL-1 β (Murray et al., 2002). A direct effect of TNF- α on cardiac hypertrophy in cultured cardiomyocytes was previously studied (Nakamura et al., 1998). TNF- α is likely to be important factors in the induction of hypertrophy (Yokoyama et al., 1997; Sekiguchi et al., 2004). In the present study, ISO intoxication significantly increased TNF- α expressions reflecting amplified inflammatory response. SL pretreatment significantly reduced its expression, indicating an anti-inflammatory effect. Our results coincided with previous studies that have reported that SL reduced the pro-inflammatory cytokines in macrophages, visceral adipose tissue, and atherosclerotic plaques (Vittone et al., 2012).

Conclusion

Taken together, the study indicate that sitagliptin, pretreatment could reduce myocardial injury and improve cardiac function in ISO induced myocardial injury by reducing oxidative damage, TNF- α , energy decline.

No conflicts of interest.

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الملخص العربي

الآلية الوقائية لدواء السيتاجلبيتين ضد إصابة عضلة القلب الناتجة عن الايزوبروتيرينول في الجرذان

وسام البقلي^١

إن دواء السيتاجلبيتين (هو أحد أدوية مرض السكر والتي تعمل كمثبط للأنزيم الدايببتيديل بيبتيداز) يقلل من الدهون الثلاثية والدهون ذات الكثافة القليلة بالدم وضغط الدم مما يساعد علي تقليل التعرض لإصابات القلب.

تهدف هذه الدراسة التي يعمل بها في

الطريقة: (/ /) في

الايزوبروتيرينول في البريتوني مرتين بجرعة حجم / في

النتائج: الايزوبروتيرينول في وجود تغيرات كبيرة ، والعديد من التغيرات في

(-) . وهذه التغيرات إلى حد كبير مع

كما يتضح من انخفاض كبير في مستوى الجلوتاثيون ديسموتاز الفائق مع زيادة في

/ايزوبروتيرينول كبير في هذه

ولقد تسبب الايزوبروتيرينول في انخفاض الطاقة بقياس (ATP/ADP) التهابات بزيادة تعبير

القلب لعامل نخر الورم /ايزوبروتيرينول في هذه القيم بالمقارنة مع الايزوبروتيرينول.

الاستنتاج: هذه النتائج تشير إلى (له قدره وقائية من إصابة عضلة القلب الناتجة عن

الايزوبروتيرينول عامل نخر الورم

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