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Effects of The Anti-glucagon Treatment (Amylin) on Isolated Hearts Performance in Experimentally Induced Type II Diabetes in Rats

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- Antiglucagon
- Amylin
- Cardiac performance
- Diabetes
- Isolated hearts.

Abstract

Background/Aims: Patients with type II diabetes (T2D) have underlying pathophysiological mechanisms that increase their cardiovascular disease risk. Anti-glucagon medications as amylin and its analogs are emerging antihyperglycemic agents which recently have gained attention. However, studies exploring cardiovascular effects of amylin have shown mixed outcomes. We, therefore, aimed to assess the effects of amylin on cardiac performance in experimentally induced T2D in rats. **Methods:** This study was carried out in a total duration of 5 weeks on 40 adult female Wistar Albino rats were allocated into 3 groups (control group, diabetic group and a group of diabetic rats treated with amylin). Rats in the diabetic and amylin treated groups were rendered diabetic by receiving high fat diet for 2 weeks, then subjected to a single intraperitoneal (i.p.) injection of streptozotocin (STZ) in a dose of 35 mg/Kg dissolved in 1 mL of 0.05 M citrate buffer. Rats in the amylin group were treated with amylin which was started at the fifth week in a dose of 20 µg/Kg once daily for 7 days. ECG as well as the in vitro responses of isolated hearts to isoproterenol infusion by Langendorff's preparation were also assessed. Blood samples were collected for biochemical measurements of fasting blood glucose, plasma insulin, glucagon, HbA1c and serum lipid profile. **Results:** Median baseline peak developed tension (PT) and PT per left ventricular weight (PT/LV) were significantly lowered in the diabetic group compared to the control group. Both parameters in the amylin treated group were significantly increased compared to the diabetic group and approached the normal control values. As regards cardiac responses to isoproterenol infusion, maximal and delta changes of heart rate (HR), PT and PT/LV were significantly decreased in the diabetic group compared to the control group. Whereas these parameters were significantly elevated in the amylin treated group compared to the diabetic group. Maximal and recovery HR values as well as maximal PT and PT/LV became normalized in the amylin treated group. The diabetic group also showed significant prolongation of time to peak tension (TPT), half relaxation time (HRT) and decrease of tension generation per unit time (TGPT), myocardial flow rate per left ventricular weight (MFR/LV) maximal responses to isoproterenol compared to control group. Those parameters were significantly improved in the amylin treated group and reached the control values, but the maximal responses of MFR/LV remained significantly lowered compared to the control group. Biochemically, amylin treatment lowered plasma glucagon level compared to diabetic and to control groups but did not increase plasma insulin level compared to diabetic group and remained significantly lowered compared to control group. **Conclusions:** Amylin, the anti-glucagon therapy, was able to impose partial improvement on cardiac chronotropic, inotropic functions and myocardial blood flow in diabetic state in response to beta adrenergic stimulation. Though, this improvement did not reach control levels and could be accounted for by glucagon lowering rather that due to insulin dependent mechanisms.

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INTRODUCTION

Over the last 20 years, the prevalence of T2D has tripled in Egypt which may be attributed to increased prevalence of obesity, lack of exercise and change in eating habits. The striking observation is that more than 40% of Egyptian patients with T2D are likely undiagnosed. According to the international diabetes federation report released in 2019, the prevalence of T2D in Egypt is more than 8 million adult patients (15.2%) rendering Egypt among the world top 10 countries in the number of patients with diabetes mellitus (DM) (1).

The risk of coronary heart disease among patients with T2D is 2-4 times higher compared to non-diabetics (2). Prolonged hyperglycemia and insulin resistance lead to development of diabetic cardiomyopathy independent of coronary artery disease (3). The prognosis for patients with T2D after myocardial infarction is worse compared to non-diabetic patients with the same infarct size (4). Metabolic and/or oxidative stress are key players in developing diabetic cardiomyopathy. Features of diabetic cardiomyopathy are stress-activated apoptotic cell death resulting in cardiac hypertrophy, collagen deposition, interstitial fibrosis and heart failure (5). Also, patients with T2D have underlying pathophysiological mechanisms that increase their cardiovascular disease risk including endothelial dysfunction, alteration in cardiomyocytes structural proteins, disruption in calcium handling, impaired cardiac contractility, neovascularization, accelerated atherosclerosis and hypercoagulable state. Neovascularization leads to macro- and microvascular complications (6).

Despite advances in treatment options for DM, there is a growing need for antihyperglycemic agents which may have also cardioprotective effects. Amylin, being an anti-glucagon therapy is an emerging antihyperglycemic agent which has gained attention as it affects glucose control through several mechanisms, including reduction of food intake, slowed gastric emptying and regulation of postprandial glucagon (7). However, studies exploring cardiovascular effects of amylin have shown mixed cardiac outcomes (8), (9). Therefore, this study was designed to assess the effects of amylin on cardiac performance in experimentally induced type 2 diabetic rats.

MATERIALS AND METHODS

Study design and experimental protocol

This study was carried out on 40 adult female Wistar Albino rats, initially weighing 150-180 g. Female rats were chosen as glucose intolerance and insulin resistance are more pronounced in them compared to male rats (10), (11). Rats were left for a period of 2 weeks for acclimatization before starting the experiments. The rats were placed in plastic cages (5-6 rats/cage). The diet was introduced daily at 8:00 AM and water ad libitum. Animals were maintained at room temperature (22 ± 1 °C) with 12 h light dark cycle. Rats were allocated into 3 groups (control group, diabetic group and a group of diabetic rats treated with amylin). Rats in control group were fed regular diet for 5 weeks and subjected to a single i.p. injection of citrate buffer (1 mL/Kg) after the 2nd week. Rats in the diabetic groups were rendered diabetic by receiving high fat diet for 5 weeks and subjected to

a single i.p. injection of streptozotocin (STZ) which was purchased from Sigma-Aldrich, Inc., USA in a dose of 35 mg/Kg dissolved in 1 mL of 0.05 M citrate buffer after the 2nd week (12). Blood glucose was tested weekly, starting from the 3rd week, using rat tail blood sample. Rats having fasting blood glucose levels ≥ 200 mg/dL were considered diabetic and were included in the study, while rats having fasting blood glucose levels lower than 200 mg/dL were excluded from the study. Blood glucose concentration was determined by Gluco-Star 2 blood glucose test strips, supplied by Taidoc Technology Corporation, Taiwan.

Rats in the amylin group were rendered diabetic by the same method and after injection of STZ (at the end of the 2nd week), rats were maintained on high fat diet for another 3 weeks with concomitant treatment with amylin rat H-9475 (which was purchased from Bachem, Bubendorf, Switzerland and subcutaneously injected at the start of the 5th week) in a dose of 20 µg/Kg once daily for 7 successive days (13). This study was approved by the Ethics Committee of Faculty of Medicine, Ain Shams University, Cairo, Egypt. Animal experimentation was performed in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academic Press).

Preparation of dietary formula

The high fat diet consisted of 57.3% fat, 24.6% carbohydrate, and 14.1% protein, in 100 g of dry food, with a total caloric value of 670.5 Kcal/100 g dry food (14), while the standard (control) diet was composed of 5.85% fat, 67.3% carbohydrate, and 23% protein, in 100 g of dry food, with a total caloric value (metabolizable energy) of 413.85 Kcal/100 g dry food (15).

Experimental procedure

On the day of sacrifice, blood glucose was measured in the overnight fasted rats. Rats were weighed and then injected i.p. with 5000 IU/Kg heparin sodium. Fifteen minutes later, they were anaesthetized with i.p. injection of thiopental sodium, in a dose of 40 mg/Kg, then, electrocardiogram (ECG) was recorded, blood samples were collected by cannulation of abdominal aorta for later determination of fasting blood glucose, plasma glucagon, fasting insulin, HbA1c and total lipid profile. Blood was collected in 3 tubes: 1. Ethylenediaminetetraacetic acid (EDTA) containing tube for subsequent determination of HbA1C in whole blood sample. 2. Heparinized tube, which was centrifuged at 4000 rpm for 15 minutes, the plasma from heparinized tube was then pipetted into clean storage labeled aliquots then stored at -80 °C for later determination of plasma insulin and glucagon levels. 3. Serum separator tube which was centrifuged at 4000 rpm for 15 minutes, the serum was then pipetted into clean storage labeled aliquots then stored at -80 °C for later determination of total lipid profile**.** After blood collection, in vitro study of isolated hearts was performed using a Langendorff's preparation to record activity of the heart under basal condition and in response to isoproterenol. Myocardial perfusate collection was performed for determination of myocardial flow rate. Body and cardiac weights were also estimated.

Preparation and perfusion technique of the isolated hearts

Hearts were removed quickly and immediately placed in ice-cold modified Krebs-Henseleit Bicarbonate (KHB) buffer solution for fast cardioplegia. The aorta was cannulated, and retrograde perfusion with fluid of about 37 °C temperature at 55 mmHg perfusion pressure was performed.

Recording of cardiac responses

Tension developed by the heart was measured by an isometric force transducer (ugo basile S.R.L., Model 7004-F, Serial N. 101014, Data EVO 14543, Italy). Isolated rat heart apex was attached by a clip to the transducer. The transducer was connected via a USB interface to the data capsule–Evo four channel digital recorder (ugo basile S.R.L. Biological Research Apparatus 21036, Model 17304, Serial N. O448A15, Italy) and to a computer provided with iWorx LabScribe2 TM Data Recording and Analysis Software. The heart was left to stabilize for 15 minutes, and then the baseline cardiac activities were recorded including HR, PT, TGPT and HRT. Myocardial flow rate (MFR) was determined by collecting the fluid passing out of the heart in glass beaker by the end of 3 minutes (timed volumetric collection), also PT/LV, TGPT and MFR/LV were calculated.

After recording of basal cardiac activities, isoproterenol was infused through a catheter tube (PE-50, Clay Adams, New Jersey). Isoproterenol was weighed and dissolved in KHB buffer to obtain a final concentration of 2 µg/mL that infused using a Sega-355 infusion pump at 5 sequential rates of 0.324, 0.648, 0.864, 1.296 and 1.728 mL/minute, to obtain final 5 doses of 0.65, 1.3, 1.73, 2.6, and 3.46 µg/minute. Each dose was infused for three minutes and then the recording was obtained, and the myocardial flow was collected and MFR was measured. Three minutes after infusion of the last dose of isoproterenol, another record was obtained to assess cardiac recovery.

Biochemical analysis

The fasting blood glucose level was determined by using Gluco-Star 2 blood glucose monitoring system (TaiDoc Technology Corporation, Taiwan). Results were expressed as mg/dL. Serum insulin was estimated using enzyme-linked immunosorbent assay (ELISA) kit supplied by Perfect Ease Biotech (Beijing) Co., Ltd., China using the quantitative sandwich enzyme immunoassay technique (16). Insulin resistance was calculated by homeostasis model assessment for insulin resistance (HOMA-IR index) according to the formula: HOMA-IR = fasting plasma insulin (µIU/mL) x fasting plasma glucose (mmol/L) /22.5 (17).

HbA1c was estimated using a test reagent kit (Bio diagnostic Company, Egypt) using enzymatic colorimetric method (18). Glucagon immunoassay was estimated by Quantikine ELISA supplied by Bio-Techne Ltd., USA using the quantitative sandwich enzyme immunoassay technique (19).

Serum triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol were estimated by enzymatic colorimetric method using diagnostic kits supplied by Bio diagnostic Company, Egypt (20).

Histologic examination

Specimens from the apex of the left ventricle of the heart were immediately fixed in

10% formalin, processed, and embedded in paraffin for light microscopic examination. Tissues were sectioned at 5 µm and stained with hematoxylin and eosin (H&E). Histopathological imaging was performed using microscope Leica DM 750/4 and digital camera Leica DFC 420 (Germany). For transmission electron microscopy, the specimens were fixed in cold 3% glutaraldehyde solution. Then post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and then examined using TEP 1010-EXII (Joel, Tokyo, Japan) at the Regional Mycology and Biotechnology Unit, Al Azhar University, Cairo, Egypt.

Statistical analysis

One-way ANOVA was used to analyze differences between means of different groups for body weight (BW) and body mass index (BMI). Wilcoxon Mann-Whitney test was used to assess differences of non-parametric variables (baseline values, maximal response to isoproterenol, delta changes of maximal responses from baseline values and recovery values of HR, PT, PT/LV, TGPT, TPT, HRT and MFR/LV between different studied groups. Pearson correlation studies were performed between different parameters. $P \leq 0.05$ was considered statistically significant. All analyses were conducted using SPSS 25.

RESULTS

Body weight and body mass index (Table 1)

Our findings revealed that initial body weight (IBW), initial body mass index (IBMI) and initial waist circumference (IWC) were nonsignificant among different studied groups.

Final body weight (FBW), final body mass index (FBMI) and final waist circumference (FWC) were significantly decreased in the diabetic group and amylin treated group compared to the control group. FBW and FBMI were significantly increased in the amylin treated group compared to the diabetic group while, FWC showed nonsignificant difference between both groups.

Cardiac weights (Table 2)

Relative cardiac weights in diabetic group showed significant increase in atria (AT)/BW, right ventricle (RV)/BW, left ventricle (LV)/BW and whole heart (WH)/BW compared with control group. Amylin treated group showed significant decrease in AT/BW, LV/BW and WH/BW compared with diabetic group, but still significantly elevated compared with control group. Absolute weights of AT, RV, LV and WH showed insignificant differences among different studied groups.

Electrocardiographic parameters (Table 3)

In this study, HR in the diabetic and amylin treated groups showed significant elevation compared to the control group. Meanwhile, HR was not significantly different between the amylin treated group and the diabetic group.

Mean values of QRS voltage in the diabetic group and amylin treated group were significantly decreased and Q-Tc were significantly increased compared to the control group. On the other hand, the amylin treated group did not show significant differences regarding both parameters compared to the diabetic group, while Q-To was not significantly different among all studied groups.

In vitro heart beating rate (HR) (Table 4)

Baseline HR in the diabetic group showed nonsignificant difference compared to the control group. Meanwhile, baseline value in the amylin

treated group was significantly higher compared to diabetic and control groups.

As regards cardiac response to isoproterenol infusion maximal, recovery values, and delta changes of HR, they were significantly decreased in the diabetic group compared to the control group. Whereas these parameters were significantly elevated in the amylin treated group compared to the diabetic group.

Median maximal and recovery HR values became normalized in the amylin treated group and showed nonsignificant difference compared to the control group. However, delta changes remained significantly decreased in this group compared to the control group.

In vitro study of inotropic baseline parameters and their responses to isoproterenol (Tables 5-8)

Baseline values of PT and PT/LV were significantly declined in the diabetic group compared to the control group. While the amylin treated group showed significant increase of both parameters compared to diabetic group, becoming non-significant compared to control group values.

Maximal values and delta changes of PT and PT/LV were significantly decreased in the diabetic group compared to the control group. Meanwhile, the amylin treated group showed significant elevation in maximal values compared to the diabetic group and reached control values, but the delta changes were not significantly different between the amylin treated group and the diabetic group, therefore, it remained significantly lowered compared to the control group.

Recovery values of PT and PT/LV were significantly decreased in the diabetic group compared to the control group. Whereas these parameters were significantly increased in the amylin treated group compared to the diabetic group but did not reach the control values and remained significantly declined compared to the control group.

Baseline TPT showed nonsignificant differences among all studied groups. On the other hand, basal TGPT was non significantly different in the diabetic group and the control group, but baseline TGPT in the amylin treated group was significantly elevated compared to the diabetic group, though, non-significant compared to control values.

As regards maximal values of TPT, the diabetic group showed significant prolongation compared to control group, meanwhile maximal values of TGPT in diabetic group were significantly decreased compared to control group. Maximal values of TPT in the amylin treated group were significantly shortened compared to the diabetic group. On the other hand, maximal values of TGPT in the amylin treated group were significantly higher compared to the diabetic group. Maximal values of TPT and TGPT were normalized and non-significantly changed compared to control group.

Moreover, the delta changes of TPT and TGPT were significantly decreased in the diabetic group compared to control group. Delta changes of TPT in amylin treated group were non-significantly changed compared to diabetic and control groups. While delta changes of TGPT in amylin treated group were non-significantly changed compared to diabetic group, delta changes of TGPT remained significantly lowered in amylin treated group compared to control group.

Diabetic group showed significant prolongation of TPT recovery values and

significant decline of TGPT compared to control group. Meanwhile, recovery values of TPT in the amylin treated group showed non-significant difference compared to the diabetic group, so, this value reached control level and became normalized. Although, recovery values of TGPT in the amylin treated group were significantly elevated compared to the diabetic group, but they remained significantly decreased compared to the control group.

In vitro half relaxation time (HRT) (Table 9)

Baseline HRT in the diabetic group showed nonsignificant difference compared to the control group. Also, baseline value in the amylin treated group was insignificantly changed compared to the diabetic group, but significantly shortened in the amylin treated group compared to the control group.

Meanwhile, maximal values of HRT were significantly prolonged and delta changes were significantly decreased in the diabetic group compared to the control group. On the other hand, maximal values of HRT in the amylin treated group were significantly shortened but delta changes were non-significantly changed compared to the diabetic group. Although, maximal values of HRT in the amylin treated group were non significantly different compared to the control group, delta changes remained significantly lowered.

Our findings revealed also that recovery values of HRT showed non-significant changes among all studied groups.

In vitro myocardial flow rate per left ventricular weight (MFR/LV) (Table 10)

Diabetic group showed significant decrease of MFR/LV regarding baseline values, maximal values, delta changes and recovery values compared to control group.

On the other hand, amylin treated group showed nonsignificant difference of MFR/LV baseline values compared to diabetic group and remained significantly lowered compared to control group. Whereas, maximal values, delta changes and recovery values of MFR/LV were significantly increased in the amylin treated group compared to the diabetic group. Maximal values and delta changes remained significantly decreased in amylin treated group compared to the control group, but recovery values became normalized and reached control values.

Table 1. Mean ±SEM of initial body weight (IBW), initial body mass index (IBMI), initial waist circumference (IWC), final body weight (FBW), final body mass index (FBMI), final waist circumference (FWC), body weight percent change (BW%), body mass index percent change (BMI%) and waist circumference percent change (WC%) in all studied groups.

	Control	**Diabetic**	**Amylin treated**
IBW (gm)	167.14 ± 2.80	163.46 ± 2.90	170.76 ± 2.20
IBMI (gm/cm2)	0.40 ± 0.01	0.37 ± 0.01	0.41 ± 0.01
IWC (cm)	12.96 ± 0.19	12.76 ± 0.16	12.76 ± 0.13
FBW (gm)	268.57 ± 3.40	180.00 ± 4.40[a]	191.92 ± 4.40[ab]
FBMI (gm/cm2)	0.64 ± 0.01	0.41 ± 0.01[a]	0.45 ± 0.01[ab]
FWC (cm)	14.00 ± 0.19	13.00 ± 0.20[a]	13.20 ± 0.17[a]
BW%	61.30 ± 3.52	10.35 ± 2.73[a]	12.51 ± 2.61[a]
BMI %	61.45 ± 3.51	10.06 ± 2.73[a]	12.09 ± 2.61[a]
WC%	8.09 ± 1.18	1.80 ± 0.82[a]	3.92 ± 0.88[a]

[a] Significance calculated by LSD at *P*-value ≤0.05 from control group

[b] Significance calculated by LSD at *P*-value ≤0.05 between diabetic group and amylin treated group

Table 2. Mean ±SEM of absolute weight (mg) of atria (AT), right ventricle (RV), left ventricle (LV), whole heart (WH), and their relative weights (mg/g) (AT/BW), (RV/BW), (LV/BW), (WH/BW) in all studied groups.

	Control	**Diabetic**	**Amylin treated**
AT	208.16 ± 2.35	206.45 ± 2.26	204.30 ± 1.67
AT/BW	0.77 ± 0.01	1.14 ± 0.09[a]	1.06 ± 0.02[ab]
RV	63.76 ± 2.43	57.16 ± 2.88	57.72 ± 2.73
RV/BW	0.23 ± 0.01	0.31 ± 0.01[a]	0.30 ± 0.01[a]
LV	411.52 ± 6.98	413.32 ± 5.32	413.27 ± 4.23
LV/BW	1.53 ± 0.02	2.29 ± 0.06[a]	2.16 ± 0.05[ab]
WH	683.45 ± 10.03	676.95 ± 9.64	675.31 ± 6.96
WH/BW	2.55 ± 0.04	3.75 ± 0.09[a]	3.31 ± 0.08[ab]

[a] Significance calculated by LSD at *P*-value ≤0.05 from control group

[b] Significance calculated by LSD at *P*-value ≤0.05 between diabetic group and amylin treated group

Table 3. Mean ± SEM of electrocardiographic changes in all studied groups.

	Control	**Diabetic**	**Amylin treated**
Heart rate (bpm)	200.86 ± 6.70	258.77 ± 7.00[a]	249.38 ± 7.50[a]
QRS voltage (µv)	0.75 ± 0.04	0.45 ± 0.05[a]	0.58 ± 0.04[a]
Q-To interval (msec.)	137.14 ± 6.90	147.69 ± 6.90	150.76 ± 4.80
Q-Tc interval (msec.)	251.28 ± 13.80	306.38 ± 14.70[a]	307.00 ± 10.50[a]

[a] Significance calculated by LSD at *P*-value ≤0.05 from control group

[b] Significance calculated by LSD at *P*-value ≤0.05 between diabetic group and amylin treated group

Table 4. Median and Interquartile range of HR (bpm), baseline values, maximal responses to isoproterenol (ISO), delta changes of maximal responses from baseline and recovery values of perfused hearts isolated from all studied groups.

Baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	89.00	(78.75 – 108.30)		
Diabetic	13	74.00	(60.50 – 174.00)	NS	
Amylin Treated	13	123.00	(98.50 – 234.50)	< 0.01	< 0.05
Maximal responses to ISO					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	166.00	(145.00 – 198.80)		
Diabetic	13	63.00	(46.50 – 121.50)	< 0.01	
Amylin Treated	13	164.00	(114.50 – 251.00)	NS	< 0.01
Delta changes of maximal responses from baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	79.50	(56.50 – 100.30)		
Diabetic	13	-15.00	(-27.50 – -6.00)	< 0.01	
Amylin Treated	13	22.00	(-23.00 – 46.50)	< 0.01	< 0.05
Recovery values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	74.50	(29.25 – 86.00)		
Diabetic	8	21.50	(7.25 – 30.50)	< 0.05	
Amylin Treated	12	48.50	(40.00 – 60.50)	NS	< 0.01

**P*-value ≤0.05 using Wilcoxon Mann-Whitney test.

*NS: non-significant.

*N: is the number of observations.

Table 5. Median and Interquartile range of PT (g), baseline values, maximal responses to isoproterenol (ISO), delta changes of maximal responses from baseline and recovery values of perfused hearts isolated from all studied groups.

Baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	1.80	(1.47 – 2.34)		
Diabetic	13	1.30	(0.65 – 1.80)	< 0.05	
Amylin Treated	13	1.80	(1.50 – 2.65)	NS	< 0.05
Maximal responses to ISO					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	2.80	(2.00 – 3.80)		
Diabetic	13	1.00	(0.65 – 1.45)	< 0.01	
Amylin Treated	13	2.30	(1.75 – 3.20)	NS	< 0.01
Delta changes of maximal responses from baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	0.80	(0.60 – 1.40)		
Diabetic	13	-0.20	(-0.40 – 0.00)	< 0.01	
Amylin Treated	13	0.01	(-0.15 – 0.75)	< 0.05	NS
Recovery values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	1.80	(1.35 – 2.10)		
Diabetic	8	0.30	(0.12 – 0.55)	< 0.01	
Amylin Treated	12	0.90	(0.40 – 1.25)	< 0.01	< 0.05

*P-value ≤0.05 using Wilcoxon Mann-Whitney test. *NS: non-significant.
*N: is the number of observations.

Table 6. Median and Interquartile range of PT/LV (g/100mg), baseline values, maximal responses to isoproterenol (ISO), delta changes of maximal responses from baseline and recovery values of perfused hearts isolated from all studied groups.

Baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	0.44	(0.38 – 0.55)		
Diabetic	13	0.31	(0.16 – 0.42)	<0.05	
Amylin Treated	13	0.45	(0.38 – 0.63)	NS	<0.05
Maximal responses to ISO					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	0.64	(0.54 – 0.90)		
Diabetic	13	0.23	(0.15 – 0.36)	<0.01	
Amylin Treated	13	0.55	(0.42 – 0.79)	NS	<0.01
Delta changes of maximal responses from baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	0.17	(0.15 – 0.34)		
Diabetic	13	-0.05	(-0.09 – 0.00)	<0.01	
Amylin Treated	13	0.00	(-0.04 – 0.18)	<0.05	NS
Recovery values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	0.43	(0.33 – 0.50)		
Diabetic	13	0.07	(0.03 – 0.13)	<0.01	
Amylin Treated	13	0.21	(0.09 – 0.32)	<0.01	<0.05

*P-value ≤0.05 using Wilcoxon Mann-Whitney test. *NS: non-significant.
*N: is the number of observations.

Table 7. Median and Interquartile range of TPT (msec), baseline values, maximal responses to isoproterenol (ISO), delta changes of maximal responses from baseline and recovery values of perfused hearts isolated from all studied groups.

Baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	137.50	(88.75 – 170.00)		
Diabetic	13	110.00	(97.50 – 132.50)	NS	
Amylin Treated	13	110.00	(90.00 – 135.00)	NS	NS
Maximal responses to ISO					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	87.50	(78.75 – 122.50)		
Diabetic	13	125.00	(100.00 – 140.00)	< 0.05	
Amylin Treated	13	90.00	(75.00 – 120.00)	NS	< 0.05
Delta changes of maximal responses from baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	-40.00	(-50.00 – -8.80)		
Diabetic	13	10.00	(0.00 – 20.00)	< 0.01	
Amylin Treated	13	-10.00	(-35.00 – 10.00)	NS	NS
Recovery values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	147.50	(127.50 – 176.30)		
Diabetic	8	187.50	(147.50 – 396.25)	< 0.05	
Amylin Treated	12	157.50	(115.00 – 175.00)	NS	NS

*P-value ≤0.05 using Wilcoxon Mann-Whitney test. *NS: non-significant.
*N: is the number of observations.

Table 8. Median and Interquartile range of TGT (mg/msec), baseline values, maximal responses to isoproterenol (ISO), delta changes of maximal responses from baseline and recovery values of perfused hearts isolated from all studied groups.

Baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	14.60	(10.61 – 16.80)		
Diabetic	13	11.80	(6.96 – 16.79)	NS	
Amylin Treated	13	16.20	(11.45 – 27.38)	NS	< 0.05
Maximal responses to ISO					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	24.40	(17.81 – 40.00)		
Diabetic	13	8.40	(4.64 – 10.84)	< 0.01	
Amylin Treated	13	22.10	(15.27 – 30.70)	NS	< 0.01
Delta changes of maximal responses from baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	11.50	(7.66 – 20.20)		
Diabetic	13	-2.70	(-4.72 – -0.51)	< 0.01	
Amylin Treated	13	1.10	(-6.84 – 13.15)	< 0.01	NS
Recovery values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	11.40	(8.27 – 15.60)		
Diabetic	8	1.40	(0.45 – 4.15)	< 0.01	
Amylin Treated	12	6.00	(2.80 – 7.86)	< 0.01	< 0.01

*P-value ≤0.05 using Wilcoxon Mann-Whitney test. *NS: non-significant.
*N: is the number of observations.

Table 9. Median and Interquartile range of HRT (msec), baseline values, maximal responses to isoproterenol (ISO), delta changes of maximal responses from baseline and recovery values of perfused hearts isolated from all studied groups.

Baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	122.50	(81.87 – 138.10)		
Diabetic	13	120.50	(70.00 – 143.75)	NS	
Amylin Treated	13	75.00	(67.50 – 96.25)	< 0.01	NS
Maximal responses to ISO					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	90.00	(61.87 – 110.60)		
Diabetic	13	130.00	(77.50 – 148.75)	< 0.05	
Amylin Treated	13	70.00	(62.50 – 97.50)	NS	< 0.05
Delta changes of maximal responses from baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	-26.30	(-33.12 – -16.30)		
Diabetic	13	5.00	(1.25 – 11.25)	< 0.01	
Amylin Treated	13	-2.50	(-10.00 – 11.25)	< 0.01	NS
Recovery values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	117.50	(93.75 – 186.90)		
Diabetic	8	178.80	(126.87 – 237.50)	NS	
Amylin Treated	12	153.80	(101.25 – 176.25)	NS	NS

**P*-value ≤0.05 using Wilcoxon Mann-Whitney test. *NS: non-significant.
*N: is the number of observations.

Table 10. Median and Interquartile range of MFR/LV (mL/min/100 mg) baseline values, maximal responses to isoproterenol (ISO), delta changes of maximal responses from baseline and recovery values of perfused hearts isolated from all studied groups.

Baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	1.15	(1.03 – 1.60)		
Diabetic	13	0.70	(0.65 – 0.90)	< 0.01	
Amylin Treated	13	0.80	(0.69 – 0.99)	< 0.01	NS
Maximal responses to ISO					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	1.60	(1.25 – 2.00)		
Diabetic	13	0.70	(0.56 – 0.84)	< 0.01	
Amylin Treated	13	0.90	(0.72 – 1.03)	< 0.01	< 0.05
Delta changes of maximal responses from baseline values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	0.20	(0.17 – 0.33)		
Diabetic	13	-0.07	(-0.08 – -0.03)	< 0.01	
Amylin Treated	13	0.10	(0.01 – 0.11)	< 0.01	< 0.01
Recovery values					
Group	**N**	**Median**	**Interquartile Range**	**P-value when compared to Control**	**P-value when compared to Diabetic**
Control	14	0.80	(0.62 – 0.93)		
Diabetic	13	0.40	(0.21 – 0.53)	< 0.05	
Amylin Treated	13	0.60	(0.41 – 0.71)	NS	< 0.05

**P*-value ≤0.05 using Wilcoxon Mann-Whitney test. *NS: non-significant.
*N: is the number of observations.

Biochemical measurements (Figures 1-3)

Plasma insulin levels in the diabetic group and amylin treated group were significantly decreased compared to the control group, while they were insignificantly changed between the amylin treated group and the diabetic group.

Fasting blood glucose, plasma glucagon, HOMA-IR score and HbA1c were significantly increased in the diabetic group compared to the control group. Meanwhile these parameters in the amylin treated group were significantly lowered compared to the diabetic group. Fasting blood glucose and HOMA-IR score in the amylin treated group were normalized and reached the control levels, while HbA1c remained significantly increased and plasma glucagon remained significantly decreased.

Serum cholesterol and triglycerides in the diabetic and amylin treated groups showed significant increase compared to the control group. Both parameters were non significantly different between amylin treated group and the diabetic group.

As regards HDL, the diabetic group showed significant decrease compared to the control group, while it was significantly higher in the amylin treated group compared to the diabetic group. HDL in the amylin treated group was non-significant compared to the control group. On the other hand, LDL showed non-significant differences among all studied groups.

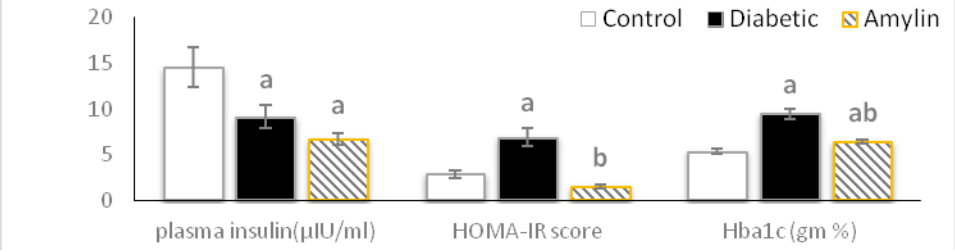
Regarding cholesterol/HDL and LDL/HDL, they were increased in the diabetic and amylin treated groups compared to control group. The increase was only significant in diabetic group, while there was significant decrease in amylin treated group compared to diabetic group.

Correlation studies in different parameters (Table 11)

Our results showed significant positive correlation between plasma insulin and HOMA-IR score but, plasma insulin was negatively correlated significantly with relative atrial and relative left ventricular weights among all groups. On the other hand, plasma glucagon showed a significant positive relationship with fasting blood glucose, HOMA-IR score, and all relative cardiac weights, while the relationship was significantly negative when plasma glucagon was correlated with FBMI among all groups.

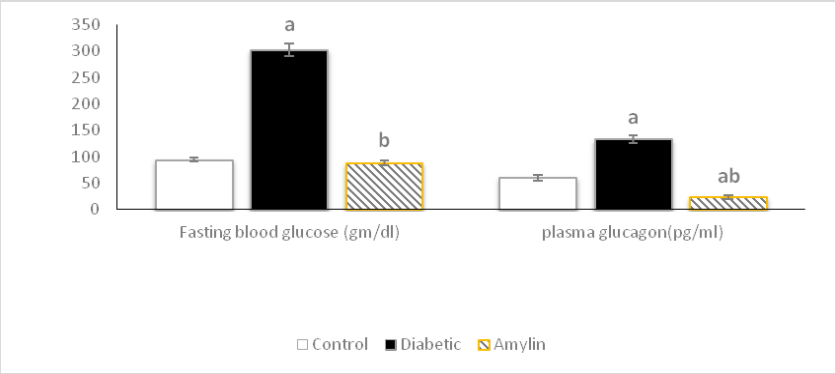
Fasting blood glucose showed an inverse relationship with FBW, FBMI, and HDL, while fasting blood glucose was positively correlated with serum triglycerides among studied groups. Additionally, HOMA-IR score showed a significant direct relationship with relative atrial weight, while HOMA-IR score was inversely and significantly correlated with delta changes of HR among studied groups.

Figure 1. Mean values of plasma insulin (µIU/mL), HOMA-IR score and HbA1c (g%) of all studied groups.



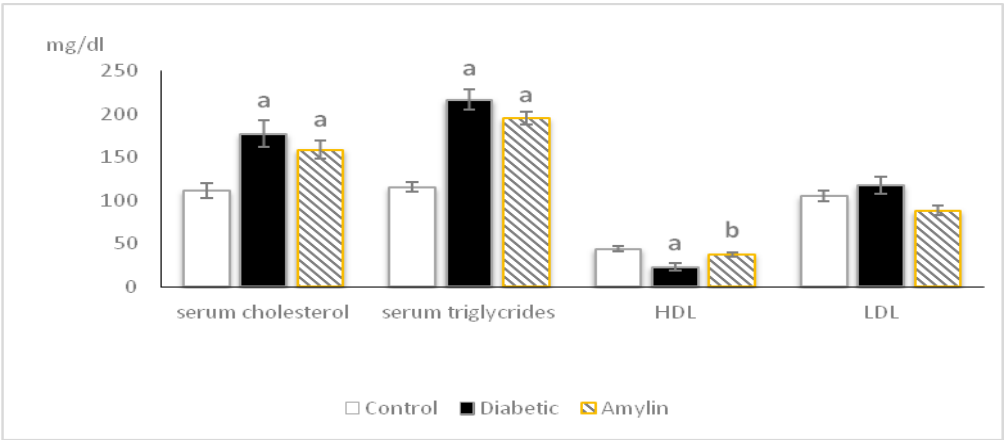
a: significance from the control group calculated using one-way ANOVA at *P*-value ≤0.05
b: significance between diabetic group and treated group calculated using one-way ANOVA at *P*-value ≤0.05

Figure 2. Mean values of fasting blood glucose (g/dL) and plasma glucagon (pg/mL) of all studied groups.

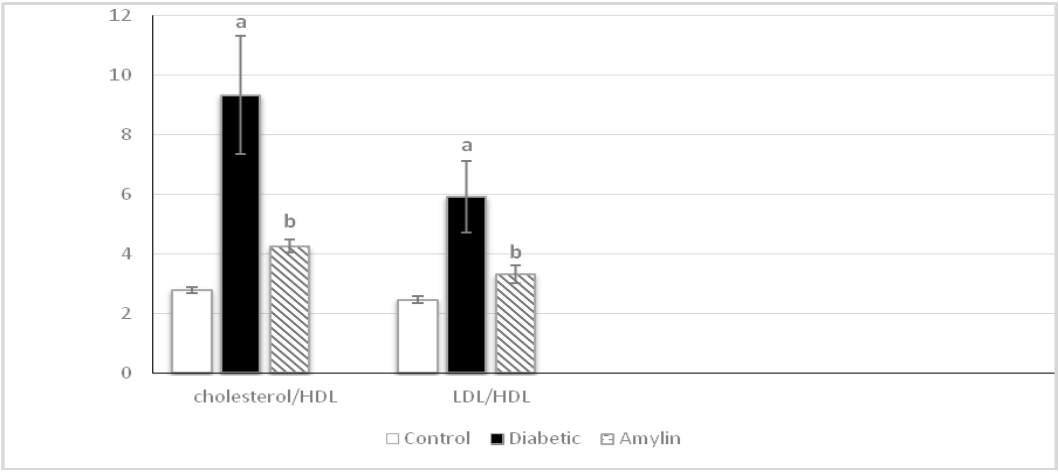


a: significance from the control group calculated using one-way ANOVA at *P*-value ≤0.05
b: significance between diabetic group and treated group calculated using one-way ANOVA at *P*-value ≤0.05

Figure 3a. Mean values of serum cholesterol (mg/dL), serum triglycerides (mg/dL), HDL (mg/dL) and LDL (mg/dL) of all studied groups.



a: significance from the control group calculated using one-way ANOVA at *P*-value ≤0.05
b: significance between diabetic group and treated group calculated using one-way ANOVA at *P*-value ≤0.05
Figure 3b. Mean values of serum cholesterol/HDL and LDL/HDL ratios of all studied groups.



a: significance from the control group calculated using one-way ANOVA at *P*-value ≤0.05
b: significance between diabetic group and treated group calculated using one-way ANOVA at *P*-value ≤0.05

Table 11. Correlation studies in different parameters

		HOMA-IR score	Relative atrial weight (mg/g)	Relative right ventricular weight (mg/g)	Relative left ventricular weight (mg/g)
Plasma insulin (μIU/mL)	r	0.382	-0.414	-0.139	-0.409
	P	<0.05	<0.05	NS	<0.05
	N	21	21	21	21

		FBMI (gm/cm^2)	Fasting blood glucose (gm/dL)	HOMA-IR score	Relative atrial weight (mg/g)	Relative left ventricular weight (mg/g)	Relative right ventricular weight (mg/g)	Relative whole heart weight (mg/g)
Plasma glucagon (pg/mL)	r	-0.42	0.812	0.764	0.475	0.445	0.553	0.482
	P	<0.05	<0.001	<0.001	<0.05	<0.05	<0.01	<0.01
	N	21	21	21	21	21	21	21

		FBW (gm)	FBMI (gm/cm^2)	Serum Triglycerides (mg/dL)	HDL (mg/dL)
Fasting blood glucose (pg/mL)	r	-0.45	-0.482	0.582	-0.647
	P	<0.01	<0.001	<0.01	<0.001
	N	40	40	21	21

		Relative atrial weight (mg/g)	HR delta changes (bpm)
HOMA-IR score	r	0.475	-0.414
	P	<0.05	<0.05
	N	21	21

*P-value ≤0.05. *NS: non-significant.
*N: is the number of observations. *r: Pearson correlation coefficients.

Histologic examination

Light microscopic examination of the control group demonstrated branching and anastomosing cardiomyocytes with focal separation detected between the cardiac muscle fibers. Nuclei were central and vesicular with most of the fibers demonstrated acidophilic striated cytoplasm and other fibers showed small vacuolations in the cytoplasm (Figure 4a). Light microscopic examination of the diabetic group demonstrated discontinuous, and irregular widely separated cardiac muscle fibers with darkly stained ad pyknotic nuclei. Some nuclei were vesicular. Cytoplasm of many fibers demonstrated vacuolations. Some fibers demonstrated moderate mononuclear cellular infiltration between the widely separated cardiac myocytes. Congestion of blood vessels was detected (Figure 4b). Light microscopic examination of the amylin treated group demonstrated widely separated cardiomyocytes with any vacuolations in the cytoplasm. Some nuclei were vesicular while others were darkly stained and pyknotic (Figure 4c).

Electron microscopic examination of the control group demonstrated euchromatic nuclei with slightly irregular nuclear envelope. The cytoplasm was filled with mitochondria variable in size and well-organized regular myofilaments forming myofibrils. However, vacuoles were detected in the cytoplasm of some fibers (Figure 5a). Electron microscopic examination of the

diabetic group demonstrated greatly affected and distorted cardiac muscle fibers. Many nuclei appeared heterochromatic and shrunken. The cytoplasm showed bizarre-shaped mitochondria variable in shape, size, and density. Some mitochondria were electron dense, and others were laminated and swollen. The myofibrils were disorganized, and the cytoplasm showed many vacuolated areas (Figure 5b). Electron microscopic examination of the amylin treated group demonstrated mild improvement. The nuclei of the cardiomyocytes appeared irregular and showed many heterochromatic regions. The mitochondria appeared variable is size and were surrounded by vacuolated areas. The myofilaments were detected with moderate arrangement (Figure 5c).

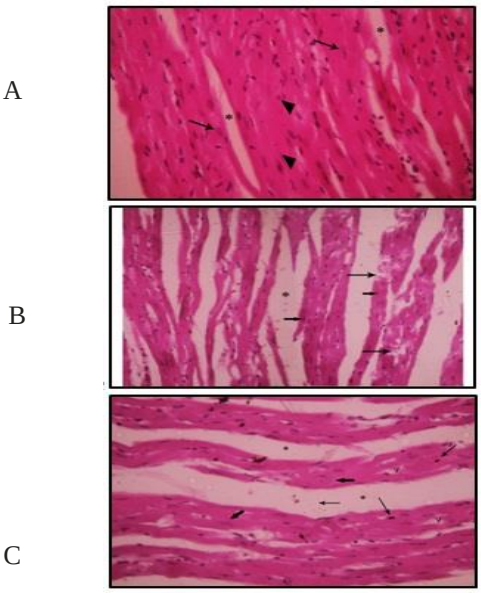


Figure 4. A) Control group, H&E, X 400 showing branching and anastomosing cardiomyocytes. Nuclei are central and vesicular (arrow). Focal separation between fibers is detected (*). Some fibers show deep acidophilic cytoplasm (a) and others show small vacuolation (arrow head). **B) Diabetic group, H&E, X 400** showing widely separated discontinuous and irregular cardiac muscle fibers. Many nuclei are darkly stained (small arrow) while others are vesicular. Vacuolation is detected in the cytoplasm (long arrow). **C) Amylin treated group, H&E, X 400** showing widely separated cardiomyocytes. Many vacuoles are detected in the cytoplasm. Some nuclei are vesicular (thick arrow) while others are darkly stained and pyknotic (thin arrow).

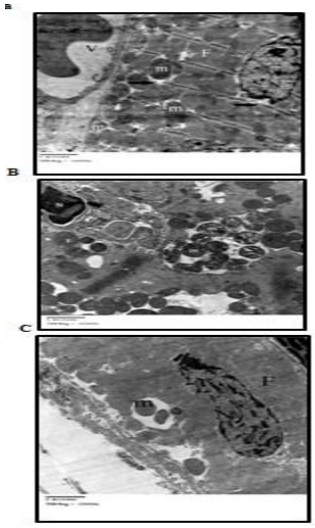


Figure 5. A) Control group, X 10000 an electron micrograph of cardiomyocyte. The nucleus (N) is euchromatic with prominent nucleolus (n). The cytoplasm is filled with mitochondria (m) variable in size and regular filaments forming myofibrils (F). Small vacuolations (arrow) are detected in the cytoplasm. Notice a nearby vessel (V). **B) Diabetic group, X 10000** showing an electron micrograph of a cardiomyocyte in the diabetic group. The nucleus is shrunken and heterochromatic (N). The cytoplasm shows disorganized myofibrils (F). The mitochondria are variable in size, shape and density. Some mitochondria show aminated cristae (m). **C) Amylin treated group, X 10000** showing the nucleus of the cardiomyocyte is irregular and shows many heterochromatic regions (N). The cytoplasm shows mitochondria (m) variable is size and surrounded by vacuolated areas (V). The myofilaments (F) are detected.

DISCUSSION

Type 2 diabetes is a complex disorder, characterized by insulin resistance, impaired glucose-stimulated insulin secretion (beta cell dysfunction) and inappropriate glucagon release (2).

Insulin has not been completely successful in counteracting the decline in pancreatic function, and ineffective in combating associated metabolic disorders. In this study, we aimed at utilizing amylin therapy (which is also co-secreted with insulin from pancreatic beta cells) to suppress glucose level as an innovative therapy for diabetes with the goal of not only controlling diabetes but also targeting improvement in metabolic as well as cardiac functions with diabetes.

STZ was reported to be a glucose analogue which is transported into pancreatic beta cells via GLUT2 glucose transporter where it accumulates and triggers necrosis and causes hyperglycemia (21). Herein, the significant increase in blood glucose level and decrease in plasma insulin level were supporting to this mechanism. Moreover, concomitant supplementation of HFD with STZ in this study is suggested to play a role in induction of hyperglycemia.

Insulin resistance usually associates high blood insulin levels. This was exhibited in our study by the significant positive correlation between blood insulin level and HOMA IR score. However, there was unexpected insulin deficiency in our study despite the high HOMA-IR score. Interestingly, hyperglycemia was concomitant with insulin deficiency induced by STZ and with insulin resistance induced by HFD as well. The aforementioned observations pointed to that our model is considered a mixture of type 1 and type 2 diabetes mellitus.

Recently, both insulin deficiency and resistance due to HFD were reported by Sakano et al (22). HFD was reported to overstimulate pancreatic beta cells and contribute to their damage leading to impairment of pancreatic function and increased risk of developing T2D (22).

In the current study, the results showed also significant decrease in FBW, FBMI and FWC of the diabetic group compared to the control group.

Weight loss may be considered a complication of diabetes as there was a significant negative correlation between fasting blood glucose and FBW, FBMI and FWC.

Diabetes causes an increase in muscle catabolism, and lipolysis and hence, decreases body weight (23). Additionally, STZ results in weight loss through induction and progression of diabetes mellitus, direct cytotoxic effects as well as due to its insulin decreasing effects (24).

Also, inability to metabolize carbohydrates as fuel sources leads to shift in reliance to fatty fuels causing wasting of fat stores and weight loss. Weight loss observed in this study with diabetes induction was reported in multiple studies either with STZ alone (25, 26) or HFD combined to STZ (27, 28).

In the amylin treated group, FBW and FBMI were significantly increased compared to the diabetic group. However, these values in amylin treated group remained low compared to control group, which could be interpreted as partial correction of diabetic condition and thereby their sequalae on body weight. Additionally, amylin

being an anti-glucagon therapy, it partially increased FBMI as noted in our study, there was significant negative interrelationship between blood glucagon level and FBMI.

Indeed, the significant increase in FBW, FBMI in amylin treated group compared to diabetic group could not be attributed to the direct effect of amylin, as amylin is reported to reduce food intake, percentage of retroperitoneal fat deposition and to decrease food intake in a dose-dependent manner (29). Moreover, amylin is described as a potential treatment for obesity and non-alcoholic fatty liver disease (30, 31). Therefore, increased weight could be attributed to the indirect effect of amylin in controlling diabetes rather than to its direct effects on appetite or on body fat composition.

Results in this study showed additionally significant decrease in plasma insulin levels in the diabetic group compared to the control group. Insulin levels vary depending on diabetes stage, as the secretory capacity of beta-cell decreases with increasing duration of diabetes (32).

This study showed that amylin treatment did not reduce plasma insulin to a significant level compared to diabetic group, a finding which denotes that amylin effects are insulin independent.

In the current work, there was significant hyperglucagonemia in the diabetic group compared to control group. Hyperglucagonemia was described with insulin deficiency in T2D (33). Patients with T2D suffer hyperglucagonemia, which worsen their hyperglycemia via stimulation of hepatic glucose production. The positive relationship between plasma glucagon and blood glucose level, encountered herein, is supported by a study which found that hyperglucagonemia is responsible for more than 50% of the inappropriate increase in blood glucose level in patients with T2D (33).

In this work, hyperglucagonemia found in the diabetic group was attributed to acute insulin withdrawal, as insulin has suppressive effect on glucagon secretion despite of hyperglycemia mediated by increased intracellular ATP in alpha cells (34). Also, it was suggested that free fatty acids have direct secretory effects on alpha cells of pancreas (35). Another study revealed that HFD results in hepatic glucagon resistance through reducing the number of hepatic glucagon receptors. As a result, hyperglucagonemia occurs to overcome hepatic glucagon resistance (36). Therefore, we suggest that HFD as well as insulin withdrawal may be the triggering factors for increasing plasma glucagon level in the diabetic group.

Moreover, in the current work, a significant decrease in plasma glucagon level was observed in the amylin treated group compared to the diabetic group. This is consistent with published literature as amylin inhibits the inappropriate release of glucagon with reduction in overall hyperglycemia (37, 38). This shows that amylin had glucagon-lowering effects in diabetic rats. Indeed, the improvement of blood glucose level in amylin treated group compared to diabetic group is assumed to be insulin independent as plasma insulin level did not show significant increase in this group compared to diabetic group. Thereby, the glucose lowering effect of amylin compared to diabetic group could be attributed to its ability to reduce glucagon level per se rather than insulin elevating effect.

HOMA-IR is significantly increased in rats rendered diabetic in our study, mirroring insulin resistance and beta cell deficiency as HOMA-IR is considered a homeostasis model assessment reflecting varying degrees of beta-cell deficiency and insulin resistance (39).

Our findings exhibited also significant reduction in HOMA-IR score in amylin-treated rats compared to diabetic group. This is explained by improvement in insulin sensitivity in addition to the tight control of blood glucose level observed herein and was denoted by the reduced HbA1c in amylin-treated group. In addition, reduced HOMA-IR was further explained by the glucagon lowering effect of amylin, as glucagon was positively correlated with HOMA-IR score and also due to the tight control of blood glucose which was shown in our study by the positive correlation between HbA1c and HOMA score.

Regarding lipid profile, this study showed significant increase in serum cholesterol, triglycerides levels and significant decrease in HDL levels in the diabetic rats compared to the controls. The triglycerides levels were positively correlated with fasting blood glucose in addition to the negative correlation between HDL and blood glucose level in the current work which reflected the direct effect of hyperglycemia in inducing dyslipidemia as blood glucose and free fatty acids serve as substrates for triglycerides production (40). This is consistent with a previous study which described diabetic dyslipidemia and its positive correlation with serum glucose and HbA1c levels (41).

Additionally, hypertriglyceridemia in diabetes may be attributed to increased secretion of VLDL and reduced clearance of triglycerides. Abnormal lipid profile can be used as a predictor of glycemic control in patients with T2D (40).

On the other hand, in the current study, the glycemic control and the glucagon lowering effects of amylin were both contributing factors in ameliorating the dyslipidemia responses to DM. Significant increase in HDL which is negatively correlated with glucagon as well as significant reduction in cholesterol/HDL ratio and significant reduction in LDL/HDL were observed in amylin treated group. In parallel, the glycemic control on lipid profile was reported by several studies. An adequate glycemic control is associated with normalization of serum levels of cholesterol, triglycerides, LDL and HDL (41, 42). Amylin was suggested to produce its lipolytic effects through stimulating the secretion of lipolytic related proteins such as leptin and lipase (43).

Regarding absolute cardiac weights, WH, AT, RV and LV weights, this study showed insignificant changes among different groups. However, significant increase in relative weights of atria (AT/BW), ventricles (RV/BW), (LV/BW) and whole hearts (WH/BW) in diabetic group compared to control group were observed. In this study, insulin was negatively correlated with relative weights of atria, left ventricle and whole heart. Such findings were also reported by another study which showed higher LV/BW in diabetic group compared to the control group and attributed these changes to increased connective tissue, perivascular fibrosis and coronary vascular wall thickening in diabetics (44). Additionally, higher WH/BW ratio was reported in diabetic rats (45). This was explained by cardiac hypertrophy in diabetics which is caused by insulin resistance, mitochondrial dysfunction, and oxidative stress

(46). In this study, there was positive correlation between HOMA-IR and relative weights of atria, left ventricle, right ventricle, and whole heart. This positive correlation is consistent with previous study which explained that insulin resistance causes overproduction of superoxide ions which are involved in genesis of hypertrophy and fibrosis (47).

Interestingly, relative cardiac weights were significantly lowered in the amylin treated rats compared to the diabetic rats, though, not normalized. The anti-glucagon effect of amylin is assumed to play a role in explaining relative cardiac weights reduction as we found a positive correlation between glucagon and relative weights which could be explained by other study that showed the cardioprotective effects of anti-glucagon treatment in preventing sequential pathological features in failing heart including cardiomyocyte hypertrophy marker gene induction and interstitial fibrosis (48). Consistently, left ventricular hypertrophy in diabetic patients regressed after control of hyperglycemia due to regression of ventricular remodeling, fibrous tissue deposition and decrease in cardiomyocytes calcium overload (49-51).

Diabetic group showed significant increase in heart rate, shortening of PR interval, prolongation of Q-Tc interval together with significant decrease in QRS duration compared to the control group. These conduction abnormalities could not be attributed to STZ as several reports have indicated that STZ does not have any cardiotoxic effect (52). However, most of these changes could be attributed to cardiac autonomic neuropathy in diabetes with increased tone of the sympathetic nervous system (53). Moreover, electrolyte disturbances can result in atrioventricular conduction abnormalities as prolonged Q-Tc interval (54). Prevalence of prolonged QT interval is higher in patients with T2D (55).

Increased heart rate and shortening of PR interval in ECG could be explained by impaired vagal tone of heart rate and/or relatively increased sympathetic control due to autonomic neuropathy (56). Autonomic neuropathy affects the longest nerve fibers and thus vagus nerve damage occurs early as it is responsible for 75% of parasympathetic activity (57).

Q-Tc which represents ventricular depolarization and repolarization was prolonged in the diabetic group. Q-Tc interval is inversely proportionate to heart rate, and so heart rate corrected QT (Q-Tc) is preferably used (58). Prolonged Q-Tc interval and autonomic dysfunction are closely correlated and are considered specific signs of autonomic cardiac dysfunction (59). Previous literature reported prolonged Q-Tc interval in diabetic patients (60) as well as diabetic rat model caused by HFD and STZ (61, 62). ECG changes as sinus tachycardia, long Q-Tc and left ventricular hypertrophy were previously reported early during DM which are in line with our results (63).

Therefore, concomitant conduction and structural changes in diabetic rats were observed in this study, as light microscopic examination showed wide cardiac muscle fibers which gives further explanation for conduction impairment denoted by the prolonged PR interval. Additionally, darkly stained and pyknotic nuclei and mononuclear cellular infiltration in addition to congested blood vessels were detected, while

electron microscopic examination revealed distorted muscle fibers, heterochromatic and shrunken nuclei, bizarre-shaped mitochondria, and many vacuolated areas in the cytoplasm.

Short QRS voltage was observed in this study which was reported in patients with type I DM (64). However, shorter QRS duration may contradict the associated increase in LV/BW ratio which could be attributed to the possibility of fibrotic changes in cardiac muscle with diabetes and the structural changes observed in light microscopic as well as electron microscopic picture in diabetic group.

Glycemic control is reported to reduce the incidence of cardiac neuropathy and slows its progression (65). Yet, in this study, amylin treatment was unable to alter the changes induced by diabetes in the ECG.

The inability of amylin treatment in slowing progression of suggested diabetic neuropathy in the heart in this study could be correlated to the finding of Almeida et al where he demonstrated the aggravation of neuropathic pain with acute subcutaneous amylin injection. Also, blocking amylin receptors by intrathecal AC187 delivery resulted in brief attenuation of neuropathic pain (66). Almeida and his colleagues suggested that brain areas involved in the cognitive-affective component of pain and descending pain modulatory pathways are activated by subcutaneous amylin and may play a role in amylin nociceptive effects (66).

In this study, we observed defective chronotropic response to all doses of isoproterenol as well as significant decrease in maximal response to isoproterenol, delta changes and recovery values in the diabetic group compared to control hearts. Reduced chronotropic activity in diabetic isolated hearts was explained by defect in control mechanisms that are either intrinsic and/or extrinsic to the heart. Several studies showed that the magnitude of negative chronotropic activity is related to duration of diabetes (67, 68). The negative correlation between HOMA-IR and delta changes of heart rate in our study could point to the involvement of insulin resistance in impairing beta-adrenergic responsiveness**.** There are conflicting data regarding the role of glucagon in heart failure with some reports investigating the use of glucagon in heart failure treatment. However, there is no sufficient evidence to suggest that elevated glucagon levels can result in heart failure or improve heart functions (69). The short duration of this study may explain the decrease (though non-significant) in basal heart rate. In our point of view, the negative chronotropic activity was attributed, at least partially, to intrinsic factors such as hyperglycemia as there was a negative correlation between fasting blood glucose level and maximal response to isoproterenol, delta changes and recovery values of HR in addition to the liability of SA node impairment in diabetics (70).

The significant decrease in response to all doses of isoproterenol in diabetic hearts could be attributed to cardiac autonomic neuropathy which plays an important role in reducing responsiveness of diabetic hearts to β-adrenergic stimulation (65). Also, these results come in line with a previous study that showed decreased response to β-adrenergic stimulation in diabetics as a result of dysregulated β-adrenergic receptors (71).

It is of interest to refer to the contradiction between the increased heart rate in ECG (in vivo)

and the decreased heart rate in isolated heart (in vitro) in the diabetic group which could be suggested to be due to the denervation of isolated hearts neglecting the in vivo effect of neuropathy without altering the intrinsic effect of hyperglycemia on SA node itself.

The blunted response of isolated heart to isoproterenol in case of DM was also reported by a previous study where steady heart rate was less responsive to exercise and stress (65).

In the current study, amylin treatment improved the chronotropic activity compared to the diabetic hearts. This was manifested by significant increase in the maximal response to isoproterenol, delta changes and recovery values in the amylin treated group compared to the diabetic group. These changes could be accounted for by improvement of hyperglycemia depending on our observation of the positive relation between blood glucose and such chronotropic parameters in addition to the supporting hypothesis of Howarth et al (70).

However, amylin treatment showed significant increase in basal heart rate compared to control and diabetic groups. This could be explained by increased excitability of cardiomyocytes in rat model with hyperamylinemia because of dysregulation in cardiomyocytes-calcium handling and pathologic remodeling (72). Another study explained that myocardial amylin accumulation increases calcium transient amplitude in cardiac myocytes in mice (73). Such mechanisms are responsible for increased excitability of cardiac myocytes and enhanced β-adrenergic response to isoproterenol (74).

Moreover, our findings revealed that the isolated hearts in diabetic rats demonstrated disrupted myocardial mechanics, including systolic dysfunction which was manifested by significant decrease in basal PT and PT/LV. In addition, there was subnormal responsiveness to B-adrenergic stimulation demonstrated by significant decrease of PT, PT/LV, TGPT and prolonged TPT. Regarding diastolic dysfunction, prolongation of HRT in response to all doses of isoproterenol was noted.

Systolic dysfunction observed in this study was explained by the reduced contractile activity caused by associated fibrotic changes in STZ induced diabetic rats observed in our histopathological examination. Also, systolic dysfunction was previously suggested to be due to diminished myofibrillar calcium sensitivity even if peak intracellular calcium was matched between diabetic and control cardiac trabeculae (75). Insulin deficiency in diabetic group played an important role in depressing cardiac contractile function as there was positive correlation between insulin and delta changes of TGPT**.** Insulin deficiency was suggested to cause activation of proteolytic pathways in heart that contributes to loss of cardiac protein (76).

On the other side, the diastolic dysfunction manifested by prolonged HRT with diabetes is explained by the prolonged sarcoplasmic reticulum calcium removal during diastole and changes in myofilaments Ca^{+2} sensitivity (77, 78).

Additionally, impairment of MFR/LV in basal condition and in response to isoproterenol was also observed in diabetic hearts. Dyslipidemia and hypercholesterolemia observed in the diabetic group may be a direct cause of coronary artery

insufficiency and hence, myocardial ischemia. T2D leads to increased hepatic production of LDL and impaired intestinal absorption of chylomicrons which results in atherosclerosis and hence increased incidence of cardiovascular disease (79). In addition, increased coronary vascular resistance was described in diabetic animals and patients; because of arteriolar thickening, capillary microaneurysms and perivascular accumulations of connective tissue (80). Also, hyperglycemia results in accumulation of advanced glycation end products reducing nitric oxide synthase activity and increased production of superoxide and eventually decreasing coronary blood flow (81).

The possible myocardial ischemia in this group can give a possible explanation for contractile dysfunction as well as abolished active Ca+2 uptake by sarcoplasmic reticulum and hence, lusitropic dysfunction (prolonged HRT). In agreement, prolonged HRT with diabetes was explained by the prolonged sarcoplasmic reticulum calcium removal during diastole and changes in myofilaments Ca^{+2} sensitivity (77, 78). These findings come in line with previous study which demonstrated reduced LV contractility, LV relaxation and increased stiffness in early stages of STZ-induced diabetic rat model (82).

On the other hand, amylin treated group showed significant increase in basal systolic function and their maximal responses to isoproterenol (PT, and PT/LV, TGPT) and significant shortening in maximal response to isoproterenol in TPT indicating better systolic function. Moreover, there was negative correlation between glucagon and maximal response to isoproterenol in PT, TGPT and PT/LV which explains the improvement in PT, TGPT and PT/LV compared to diabetic group. In addition, amylin treatment showed significant shortening in maximal response to isoproterenol in HRT indicating better diastolic function and significant increase in maximal response of MFR/LV indicating better myocardial flow compared to diabetic group. These findings are consistent with a previous study that showed positive chronotropic and inotropic effects of amylin on rat hearts which were attributed to the similarity between amylin and calcitonin gene related peptide (CGRP). CGRP increases calcium release from sarcoplasmic reticulum through ryanodine-sensitive calcium channels and improves myofibrillar calcium sensitivity (83). Also, the improved myocardial flow rate with amylin in this study seems to be due to glucose lowering effect of amylin as there was a negative correlation between fasting blood glucose and MFR/LV. Glycemic control with amylin was reported to improve endothelial function and decrease inflammatory responses (84). The glycemic control leads to improvement in insulin resistance which is shown in our study by negative correlation between HOMA-IR and maximal response to isoproterenol and delta changes in MFR/LV. Additionally, significant increase in HDL in amylin treated group compared to diabetic group are suggested to improve cardiac perfusion.

On the other hand, another study showed accumulation of amylin oligomers in cardiac myocytes increasing myocardial stiffness and altering calcium cycling that eventually result in impaired relaxation and diastolic dysfunction (72). However, these effects were only demonstrated in humans but not in mice and rats as the humans have an amyloidogenic promoting region which

form toxic aggregates when overexpressed (72). Moreover, human amylin accumulation in hearts results in cardiomyocyte sarcolemma calcium leakage, independent of diabetic cardiac remodeling (85).

CONCLUSION

At the end of this study, we concluded that induction of diabetic mellitus deteriorated the in-vitro inotropic, chronotropic and perfusion functions of the hearts. Amylin, the anti-glucagon therapy, did not alter blood insulin level, nevertheless, it was able to impose partial improvement on cardiac chronotropic, inotropic functions and myocardial flow rate in response to beta adrenergic stimulation, even though these ameliorating effects were not optimum and did not reach control levels. The cardioprotective effects of amylin were, therefore, due to glucagon lowering and/or improving insulin resistance rather than due to insulin dependent mechanisms.

RECOMMENDATION

On the background of this contradiction between better response to amylin in rat-hearts and the deleterious effects on human hearts, we recommend studying the cardiac performance in response to amylin analog, pramlintide, which shows less amyloidogenic effects.

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