THE EFFECT OF DULAGLUTIDE AND IVABRADINE ON CARDIAC DYSFUNCTION IN RATS WITH EXPERIMENTAL METABOLIC SYNDROME

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ABSTRACT

Metabolic syndrome (MS) comprises a collection of conditions that together raise the risk of coronary heart disease (CHD), insulin resistance (IR), stroke, fatty liver, cancers, and other serious health problems.

Our study aims to identify the protective effect of dulaglutide and ivabradine on MS-induced in male albino rats by adding 30% sucrose to drinking water. Rats were divided into six groups: three groups have not been sucrose-fed (control, dulaglutide, and ivabradine); and three groups have been sucrose-fed (sucrose, dulaglutide + sucrose, and ivabradine + sucrose).

Dulaglutide was given subcutaneously in a dose of (0.626 mg/kg) twice weekly. Ivabradine was given orally at a dose of (10 mg/kg) daily. The two drugs were given with sucrose and without sucrose for 3 weeks to study their effect on systolic blood pressure (SBP), heart rate (HR), and serum levels of glucose, insulin, Hb A1C, Rho kinase, LDL, and Nitric oxide (NO). The levels of brain natriuretic peptide (BNP) and proprotein convertase subtilisin/kexin type 9 (PCSK9) were evaluated in cardiac tissues. A histopathological examination of cardiac tissues was also performed.

We found that sucrose-induced MS led to a significant increase in the levels of the following: serum glucose by 400%, insulin by 150%, HbA1C by 60%, LDL by 60%, Rho by 700%, NO by 580%, and SBP by 26%. The cardiac levels of PCSK9 increased by 600%. At the same time, BNP cardiac levels decreased significantly by 62%. The histopathological examination of cardiac tissue of rats with sucrose-induced MS by light microscopy using H&E stain revealed abnormal degenerative changes, congestion, and tissue inflammation and infiltration. The cardiac tissue stained by Masson's trichrome showed a marked deposition of collagen fiber.

In sucrose-fed rats, dulaglutide caused a significant decrease in the levels of the following: serum glucose by 51%, insulin by 41%, HbA1C by 25%, LDL by 30%, Rho 63%, NO 68%, and SBP by 30% compared to untreated sucrose-fed rats. Ivabradine given to sucrose-fed rats caused a significant decrease in the levels of the following: serum glucose by 50%, insulin by 39%, HbA1C by 25%, LDL by 34%, Rho 63%, NO 74%, and SBP by 24% compared to untreated sucrose-fed rats. There was a significant decrease in PCSK9 cardiac levels with dulaglutide and ivabradine in sucrose-fed rats by 72% and 73% respectively less than in untreated sucrose-fed rats. At the same time, BNP cardiac levels increased significantly by 147% and 143% with dulaglutide and ivabradine respectively. The histopathological examination showed improvement of pathological changes in cardiac sections of the sucrose groups taking dulaglutide and ivabradine.

In conclusion, dulaglutide, and ivabradine may have a protective cardiac effect in sucrose-induced metabolic syndrome.

Keywords: Sucrose, Metabolic syndrome, Dulaglutide, Ivabradine, Rho, BNP, PCSK9.

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INTRODUCTION

Metabolic syndrome (MS) comprises a collection of conditions that together raise the risk of coronary heart disease (CHD), insulin resistance (IR), stroke, fatty liver, cancers, and other serious health problems [1]. It is a multifactorial syndrome with multiple risk factors that arise from insulin resistance accompanying abnormal adipose deposition and function [2].

MS can be induced by the over-consumption of a high-fat diet, and sucrose or fructose. Over-consumption of omega-6 fatty acids, particularly arachidonic acid (AA) and its precursor linoleic acid serve as substrates for the synthesis of inflammatory mediators, playing an important role in the pathogenesis of MS [3].

Rats fed with 33% sucrose are proposed as a model for the development of MS. Sucrose increases the levels of TG inducing visceral fat resulting in insulin resistance (IR). Chronic inflammation predisposes to an increased risk of hypertension, atherosclerosis, and DM [4].

The pathophysiology of MS is complex. The most important risk factors are fatty diet, genetics, aging, sedentary behavior, low physical activity, disrupted chronobiology/sleep, disorders of mood /psychotropic medications, stress, and excessive alcohol intake [5]. The accumulation of oxidative mitochondrial products is associated with mitochondrial dysfunction and IR [6,7].

Central obesity is both a sign and a cause of the excessive release of FFA directly to the liver that promotes gluconeogenesis, and VLDL synthesis decreases glucose uptake and causes overall IR [8]. IR establishes when a reduction in insulin action results in fasting hyperinsulinemia to keep euglycemia, even before fasting hyperinsulinemia develops, postprandial hyperinsulinemia exists [9].

A major factor in the development of IR is an abundance of circulating FFA. FFA are produced from TGs of adipose tissue via the action of the cAMP-dependent enzyme lipase and via the lipolysis of TG-rich lipoproteins in tissues through the effect of lipoprotein lipase. FFAs decrease insulin sensitivity in muscles by inhibiting insulin-mediated glucose uptake along with a reduction in the conversion of glucose to glycogen and increased TG accumulation[10]. The IR produced by excessive FFA enhances the synthesis of IL-6 and TNF-α that results in changing the levels of other substances (e.g., adiponectin, resistin); thus, increasing IR and lipolysis of adipose tissue TG stores to circulating FFA [7,9].

IR is combined with increases in uric acid, prothrombotic factors (fibrinogen, plasminogen activator inhibitor1), serum viscosity, asymmetric dimethylarginine, homocysteine, WBC pro-inflammatory cytokines (TNF-α, IL-6), and the presence of microalbuminuria [11]. The reduced production of anti-inflammatory and insulin-sensitizing cytokines, e.g., adiponectin, is associated with MS [12–14].

Pyruvate oxidation is impaired in diabetes causing an increase in intracellular glucose levels and a decrease in glycolytic intermediates that activate glucose-sensing transcription factors [15]. High glucose concentration causes downregulation of NO resulting in increased vasoconstriction. [16].

The goal of MS therapy is to increase the HDL levels to ≥ 40 mg/dL in males or 50 mg/dL in females. Lifestyle modifications, such as maintaining a healthy diet, and physical activity, are essential in management. When medical treatment becomes important, fibrates and statins are the most important in controlling MS-induced hyperlipidemia [17].

The inhibitors of angiotensin-converting enzymes or blockers of angiotensin receptors are important drugs for MS patients when associated with DM [18]. Glycemic control to an HbA1c of less than 7% will reduce microvascular complications and could decrease the risk for macrovascular disease as well [11]. Metformin decreases the risk for DM in patients with impaired glucose tolerance (IGT) or impaired fasting glucose (IFG), but will not decrease the risk for cardiovascular disease events in people with MS [19].

MS is characterized by increased fibrinogen, plasminogen activator inhibitor 1, and other coagulation factors rising the risk for arterial thrombosis in MS patients. This can be managed by using low-dose aspirin or another antiplatelet medicine if not contraindicated in these [20].

Weight reduction will reduce cytokine levels and thus can improve the inflammatory state. The drugs, however, cannot be reported exactly to decrease a proinflammatory state apart from other risk factors [21].

Sucrose is a disaccharide molecule 2 monosaccharide: glucose and fructose. Sucrose is produced naturally in plants, from which table sugar is refined. It has the molecular formula $C_{12}H_{22}O_{11}$ [22]. It is a dietary source of fructose. Thus, sucrose feeding is used to induce MS in animal models. Sucrose induced lipogenesis in rats with increased plasma concentrations of insulin, leptin, triglycerides, glucose, and FFAs, and impaired glucose tolerance. Sucrose produces many risk factors for CHD via increasing systolic BP in rats with increased left ventricular mass and MS [23,24].

The fructose overload in the liver causes excess substrate that produces increased lipogenesis. It increases the intrahepatic lipid supply directly, by synthesis of fatty acids, and indirectly, by inhibiting fatty acid oxidation [25].

Compared with glucose exposure fructose exposure, has been shown to produce activation of c-Jun NH2-terminal kinase, increased serine phosphorylation of IRS-1, and decreased insulin-

induced tyrosine phosphorylation of IRS-1 and IRS-2 in isolated hepatocytes that predispose to metabolic disease [26].

Dulaglutide is a glucagon-like peptide-1 receptor agonist (GLP-1RA) indicated to improve glycemic control in adults with T2DM together with diet and exercise [27]. Dulaglutide also increases insulin secretion when glucose levels are elevated, decreases glucagon secretion, and delays gastric emptying to lower postprandial glucose levels [28]. GLP-1 receptor agonists can be used in people with T2DM who either cannot tolerate metformin or have CVD risk factors [29]. Activation of AMP-activated protein kinase, which acts as a regulator of cellular energy status, has also been reported as the underlying mechanism for improved cardiac function in T2DM after liraglutide, as observed in the preclinical study [30,31].

Furthermore, GLP-1 receptor agonists remove the intramyocardial lipid deposition in diabetic mice, in association with ameliorated cholesterol levels in the blood [32]. PCSK9 antibodies along with GLP-1 analogs are associated with an acceptable tolerability profile and significantly decreased LDL-cholesterol levels in a dose-dependent manner in overweight or obese patients with T2DM [33].

Elevated resting heart rate (HR) is a known marker for heart failure (HF). Ivabradine acts on the If ion which is the most important ionic current for regulating pacemaker activity in the sino-atrial (SA) node that decreases the heart rate [34].

In patients with heart failure and reduced ejection fraction, a higher HR was associated with increased mortality in SR. If inhibitors (ivabradine) were found to improve outcomes in patients with chronic HF and reduced ejection fraction [35].

Swenja *et al.* (2011) studied the effects of ivabradine in Wistar rats with angiotensin-II-induced hypertension and found that ivabradine sustained a 15–20% heart rate reduction. Ivabradine has anti-oxidative effects associated with a decreased NADPH oxidase activity and prevented NOS uncoupling [36].

The plasma levels of brain natriuretic peptide (BNP) increase in patients with asymptomatic or symptomatic left ventricular dysfunction and are associated with coronary heart disease and myocardial ischemia. So, BNP plasma levels are used for screening and diagnosis of acute congestive heart failure and establishing the prognosis of heart failure [37].

Type 2 diabetes mellitus (T2DM) is associated with considerable increases in cardiovascular morbidity and mortality [38]. NO reduction in the vessel wall in DM may produce endothelium dysfunction. The bioavailability of NO is an important factor in the regulation of vascular tone [39].

LDL cholesterol, the small density LDL particles, and triglycerides are elevated in diabetic dyslipidemia, and HDL cholesterol is decreased [40].

Proprotein convertase subtilisin/kexin type-9 (PCSK9) is a circulating protein found mainly in the liver, intestine, kidneys, and brain. It impairs the LDL removal by binding directly to the epidermal growth factor repeat-A of the LDL receptor and targeting it for degradation [41]. Many studies have revealed that anti-PCSK9 therapies markedly reduce LDL levels leading to a lower incidence of adverse cardiovascular disease outcomes in high-risk patients with hyperlipidemia [42].

MATERIALS AND METHODS

The experimental protocol of the current study was approved by the Ethics Committee of Animal Research of the Faculty of Medicine, Fayoum University.

Chemicals

Dulaglutide (**Trulicity Pens®**: Elli Lilly; California, America): Each pen contained 1.5 mg/0.5 ml of dulaglutide. Each rat was injected subcutaneously with a dose of 0.626 mg/kg twice weekly for 3 weeks [27].

Ivabradine (**Procoralan Tablets®**: Servier, Paris, France): Each tablet contained 5 mg of ivabradine. The tablet was crushed and dissolved in distilled water and given orally to rats by gavage at a dose of 10 mg/kg daily for 3 weeks [36].

Sucrose (commercial markets; El Nasr Pharmaceutical Chemicals, Cairo, Egypt): Rats were given sucrose (30%) in drinking water [4].

Experimental Animals

In this study, 36 age-matched adult male albino rats of Wistar strain weighing about 180-200 g were used. The rats were obtained from the animal house of the NRI, Cairo University. All rats were put under observation for one week before the study with free access to food and water. Rats were exposed to a daily light/dark cycle. All rats were put in pairs in wire cages under good sanitary conditions and normal humidity.

In this study, rats were divided into 6 groups each containing 6 rats.

Group 1 (Control group):

Rats received distilled water orally for 3 weeks.

Group 2 (Dulaglutide group):

Rats were injected subcutaneously with dulaglutide (0.626 mg/kg) twice weekly for 3 weeks.

Group 3 (Ivabradine group):

Rats were given oral ivabradine (10 mg/kg) daily for 3 weeks.

Group 4 (Sucrose-fed group):

Rats were given sucrose (30%) in drinking water for 3 weeks.

Group 5 (Sucrose + Dulaglutide group)

Rats were given sucrose (30%) in drinking water and given a subcutaneous injection of dulaglutide (0.626 mg/kg) twice weekly for 3 weeks.

Group 6 (Sucrose + Ivabradine group)

Rats were given sucrose (30%) in drinking water and given oral ivabradine (10 mg/kg) daily for 3 weeks.

Experimental Design

At the end of the three weeks, animals were deprived of food for 12 hours and the following parameters were investigated:

1. Body Weight

The weight of all rats was measured using a weight scale by the end of the 3 weeks.

2. Heart rate and Blood pressure

By the end of every week, heart rate and blood pressure were measured by the ADI instruments IN125 NIBP (Non-invasive Blood Pressure) (Australia). The controller performs non-invasive blood pressure and pulse measurement on rats using specialized tail cuffs.

The sensitive blood pressure meter was switched on and allowed to acclimatize for about 20 min; the selector switch located at the back of the equipment was switched to the area marked for rats. The rats were introduced into the transparent glass restrainer. The animals were covered with pieces of dark clothes to the reduction of anxiety. The tail cuff/transducer was introduced into the base of the tail region and the selector switch turned on, and the readings were displayed on the screen of the apparatus [43].

Figure 1: Non-invasive Blood Pressure in rats

3. Blood sample collection and serum analysis

Blood samples (2.5-3 mL from each rat) were collected from the retro-orbital plexuses of each rat. The blood was centrifuged at 3000 rpm for 5 minutes and a Pasteur pipette was used to collect the supernatant (i.e., the serum) which was stored frozen until needed for analysis of serum glucose, insulin, Hb A1C, Rho, and NO.

Immediately after collection of blood, rats were sacrificed by cervical dislocation, hearts were excised and washed with ice-cold saline, then each heart was sectioned into two parts, one part was preserved in formalin (10%) for histopathological examination The other part was preserved in a deep freezer at -20 ^{o}C up to measure Brain natriuretic peptide (BNP) and Proprotein convertase subtilisin / kexin type 9 (PCSK9) by enzyme-linked immunosorbent assay (ELISA).

3.1. Determination of fasting serum glucose

Serum glucose was determined after enzymatic oxidation in the presence of glucose oxidase. The formed hydrogen peroxide reacts with phenol and 4-aminophenazone under the catalysis of peroxidase to form a red violet quinoneimine [44].

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + [4\text{-aminophenazone}] + \text{phenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4H_2O$$

Method:

The contents were mixed and then incubated for 10 minutes at 37 ^{o}C. The absorbance was read at 500 nm against a blank containing only 2000 µl of enzyme reagent. The same procedure was repeated using 20 µl of the standard instead of the sample.

Calculation:

$$\text{Concentration of glucose } \left(\frac{\text{mg}}{\text{dL}}\right) = 100 \times \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}$$

3.2 - Determination of serum HbA1C

Application:

This kit can be used to measure Glycosylated Hemoglobin (GHb/ Hb A1C) content in animal red blood cells, whole blood, and other samples. This kit (15 Assays) can detect 14 samples [45].

Detection significance:

Hb A1C is an index that reflects the control of blood glucose in diabetes for a long time (4–10 weeks). Poor long-term control of blood glucose will cause increased content of glycosylated hemoglobin. So, the determination of Hb A1C can help to control the blood glucose in diabetics and it plays an important role in the study of peripheral vascular and cardiovascular complications of diabetes.

Detecting principle:

The glycosylated hemoglobin with ketoamine bond in hemoglobin is heated in an acidic environment, and hexose is dewatered partially to form 5-hydroxymethylfurfural (5-HMF). 5-HMF can react with TBA to form a yellow complex which can be detected by colorimetric assay at 443 nm with a 1 cm optical path cuvette.

Calculation of results

The content of GHB is expressed in terms of absorbance per 10 g of hemoglobin.

$$\text{GHb content} = \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{Blank}}}{\text{Hemoglobin content in 2mL hemolysis}} \times \text{Dilution factor} \times 10\ \text{g}$$

$$= \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{Blank}}}{\text{Hemoglobin content /the volume of hemolysis}} \times 10$$

3.3. Determination of serum LDL

The LDL Cholesterol Assay Kit is a plate-based colorimetric assay for the determination of LDL cholesterol in serum or plasma samples [46].

The kit uses a spectrophotometric assay to detect LDL directly from serum samples, enabling researchers to measure LDL cholesterol levels in rodent serum. The unique features of the kit are:

- High sensitivity and low detection limit (10 mg/dL)
- A rapid (10 minutes) and robust enzyme-based assay which does not require expensive instrumentation
- High reproducibility

3.4 Determination of serum Nitric Oxide

Nitric Oxide (NO) is synthesized in the biological system by the enzyme Nitric Oxide Synthase (NOS). NOS is a remarkably complex enzyme that acts on molecular oxygen, arginine, and NADPH to produce NO, citrulline, and NADP +.

$$O_2 + \text{Arginine} + \text{NADPH} \xrightarrow{\text{NOS}} \text{NO} + \text{Citrulline} + \text{NADP}$$

NO is produced in trace quantities by neurons, endothelial cells, platelets, and neutrophils in response to homeostatic stimuli. This NO is scavenged rapidly ($t_{1/2}$ = 4 seconds) and acts in a paracrine fashion to transduce cellular signals. NO is also produced by other cells (macrophages, fibroblasts, hepatocytes) in micromolar concentrations in response to inflammatory or mitogenic stimuli [47]**.**

The final products of NO in vivo are nitrite (NO^{2-}) and nitrate (NO_3). The relative proportion of NO^{2-} and NO^3 produced from NO is variable. The exogenous source of NO^3 ingested in the diet should be considered and cannot be ignored (non-NO origin). Thus, one of the indices of NO production is NO_2.

The Biodiagnostic Nitrite Assay Kit provides an accurate and convenient method for the measurement of endogenous nitrite concentration as an indicator of nitric oxide production in biological fluids. It depends on the addition of Griess Reagents which convert nitrite into a deep purple azo compound, photometric measurement of the absorbance due to this azo chromophore accurately determines NO_2 concentration.

Principle:

In an acid medium and the presence of nitrite the formed nitrous acid diazotize sulphanilamide and the product are coupled with N-(1-naphthyl)

ethylenediamine. The resulting azo dye has a bright reddish-purple color which can be measured at 540 nm.

Calculation:

$$\text{Nitrite in sample } \mu\text{mol/L} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times 50$$

3.5- Biochemical parameters by ELISA

3.5.1- Determination of serum Insulin:

Insulin concentrations were measured in previously frozen and thawed serum samples by enzyme immunoassay using the Rat Insulin ELISA kits [48].

Principle of the test:

The rat insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule, during incubation insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies, and anti-insulin antibodies bound to the microtiter wells. A simple washing step removes unbound enzyme-labeled antibodies. The bound conjugate is detected by reaction with 3, 3`, 5, 5`-tetramethylbenzidine. The reaction is stopped by adding acid to give a colorimetric endpoint result that is read spectrophotometrically.

Assay procedure:

The reagents were allowed to reach room temperature approximately 25 °C and were mixed by gentle inversion before use:

25 µL of the standards or unknown were added to anti-insulin wells then 50 µL of the conjugate solution was added to the standards and the unknown. This was followed by incubation on a shaker for 2 hours at room temperature. After that washed 6 times with an automatic washer.

Then 350 µL of washing solution was added to each well and aspirated completely.

This was repeated 5 times, and after the final wash, the plate was inverted and tapped firmly against absorbent paper. 200 µL of peroxidase substrate was added to both the standards and the unknown. This was followed by incubation for 15 minutes at room temperature. 50 µL of stop solution was added and the plate was placed on the shaker for approximately 5 seconds to ensure the mixing of the substrate and stop solution. Finally, the absorbance was measured at 450 nm.

Calculation of results:

1. The absorbance values obtained for the standards were plotted against the insulin concentration on an in-log paper and a standard curve was constructed.
2. The concentrations of the unknown samples were read from the standard curve.

3.5.2- Determination of serum Rho-Associated Protein Kinase 1 (ROCK1).

This product is suitable for in vitro quantitative detection of rat serum, plasma, or cell culture supernatant and organizations in the natural and recombinant ROCK1 concentration.

This kit employs a double antibody sandwich technique. The principle of Double Antibody Sandwich is based on characteristics of the tested antigen with more than two valances which can identify coated antibody and detection antibody at the same time [49].

The specific process is as follows:

We connected the specific antibodies and solid phase carriers to form immobilized antibodies. Then washed-out uncombined antibodies and impurities. Then we sealed the rest binding sites with irrelevant proteins. Then joined under the test with immobilized antibodies for contact reaction. After a while, we combined antigens and antibodies on carriers into the antigens complex. Then washed-out uncombined antibodies and impurities. After that added biotin labeling antibodies to combine with the antigens on immune complexes. Then washed out the uncombined biotin labeling antibodies thoroughly. The enzyme amount on the carrier is now positively related to the amount of the tested substance in specimens.

Then, we added horseradish peroxidase to label the avidins and incorporate them with the biotin labeling antibodies. Wash out the incorporated enzyme markers thoroughly. The enzyme amount on the carrier is positively related to the amount of the tested substance in specimens.

Finally, we added substrates for coloring, and compute the concentration of specimens. An

antibody molecule can be marked on several biotin molecules and a biotin molecule can relate to an HRP-Avidin to form numbers of horseradish peroxidases combined with antibodies which shows higher sensitivity and amplification effect compared to traditional direct HRP-Antibodies.

3.5.3 Determination of Cardiac Brain natriuretic peptide (BNP)

Assay principles

The MyBioSource' Rat BNP ELISA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of Rat BNP in Cell Culture. This assay employs an antibody specific for Rat BNP coated on a 96-well plate. Standards and samples are pipetted into the wells and BNP present in a sample is bound to the wells by the immobilized antibody. The wells are washed and a biotinylated anti-rat BNP antibody is added. After washing away unbound biotinylated antibodies, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of BNP bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm [50].

Assay procedure

Prepare all samples and standards, add 100 mL standard or sample then wash plate 3 times with wash buffer working solution after that add 100 mL Biotin-labeled detection Antibody Working Solution then wash plate 3 times with wash buffer working solution after that add 100 mL Streptavidin-HRP working solution then wash plate 5 times with wash buffer working solution after that add 100 mL TMB Substrate solution then add 100 mL Stop solution finally read the plate at 450 nm.

3.5.4- Determination of cardiac PCSK-9 gene expression in the heart:

By using rat proprotein convertase subtilisin/kexin type 9 (PCSK9**)** ELISA Kit (according to Cusabio Technology LLC (Houston, TX77054, Unites States) Catalog Number. CSB-EL017647RA) For the quantitative determination of rat proprotein convertase subtilisin/kexin type 9 (PCSK9) concentrations in serum, plasma, tissue homogenates. By the quantitative determination of rat proprotein convertase subtilisin/kexin type 9 (PCSK9) concentrations in serum, plasma, and tissue homogenates [51].

Principle of the assay:

This assay employed the competitive inhibition enzyme immunoassay technique. The microtiter plate provided for this kit had been pre-coated with PCSK9. Standards or samples were put to the appropriate microtiter plate wells with Horseradish Peroxidase (HRP) conjugated antibody preparation specific for PCSK9. The competitive inhibition reaction was launched between pre-coated PCSK9 and PCSK9 in samples. A substrate solution was added to the wells and the color develops opposite to the amount of PCSK9 in the sample. The color development was stopped and the intensity of the color was measured.

Assay procedure:

All reagents and samples were at room temperature before use. The sample was centrifuged. Referring to the Assay Layout Sheet, the number of wells was determined to be used and any remaining wells and the desiccant back were put into the pouch, the Ziploc was sealed, and the unused wells were stored at 4 °C.

A blank well was set without any solution. 50 µL of standard and sample was added per well. 50 µL HRP-conjugate (1x) was added to each well immediately (not to the Blank well). The plate was shacked gently for 60 seconds.

A plate layout was provided to record standards and samples were assayed. The plate was covered with the adhesive strip provided, Incubated for 1 hour at 37°C. Each well was aspirated and washed, the process was repeated four times for a total of five washes by filling each well with Wash Buffer (200 µL) using a squirt bottle, multi-channel pipette, manifold dispenser, or auto washer and letting it stand for 2 minutes, complete removal of the liquid at each step was essential to good performance.

After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels. 90 µL of TMB Substrate was added to each well, incubated for 20 minutes at 37 °C, and protected

from light. 50 µL of Stop Solution was added to each well, the plate was tapped gently to ensure thorough mixing. The optical density of each well was determined within 5 minutes, using a micro plate reader set to 450 nm.

Calculation of results:

The professional soft "Curve Expert" was used to make a standard curve. A standard curve was created by reducing the data using computer software capable of generating a four-parameter logistic (4-PL) curve-fit. A standard curve was constructed by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis.

4. Histopathological Examination of Cardiac Tissue:

The hearts of the rats were excised and preserved in 10% formalin solution, after fixation, each heart was subjected to routine tissue processing by:

1. Dehydration in ascending alcohol, 70%, 90%, and 100%.
2. Clearing in xylol for 15 minutes.
3. Embedding in paraffin wax.

Serial sections of 4 microns thickness were obtained and stained with *Hematoxylin* and *Eosin* to asses myocardial histopathological changes as granulation tissue formation, contraction bands, vacuolar degenerative changes, and congestion and *Masson's trichrome* stain to demonstrate fibrosis.

Statistical Analysis:

The collected data was revised for completeness and logical consistency. Data was then transferred to the statistical package for social science (SPSS) Version 16 to be statistically analyzed by the following levels of analysis:

1. Descriptive statistics were used to express our data (mean ± standard deviation for quantitative variables).
2. Comparison of quantitative variables between groups was done using an ANOVA test followed by a post hoc test for inter- group comparison.
3. Pearson's Rank correlation was done to test the association between variables and expressed using (r).
   If r value = + 0.60 or higher → Very strong positive relationship
   = -0.60 or higher → Very strong negative relationship
4. P value < 0.05 was considered significant.

RESULTS

A. Hemodynamic parameters

1. Systolic blood pressure (SBP):

Our study showed that the SBP was 131.67 ± 2.58 mmHg in the normal control group.

In the dulaglutide and ivabradine control groups SBP was 121.3 ± 6.47 and 115.00 ± 5.58 mmHg respectively. In the sucrose group, SBP was 166.5 ± 12.32 mmHg (Figure 2, Figure 3, Figure 4, Figure 5).

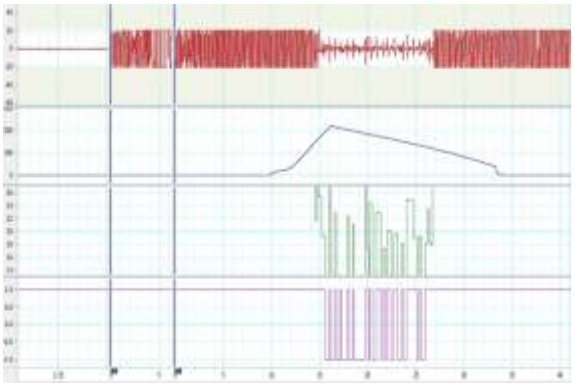

Figure 2: BP in the normal control group

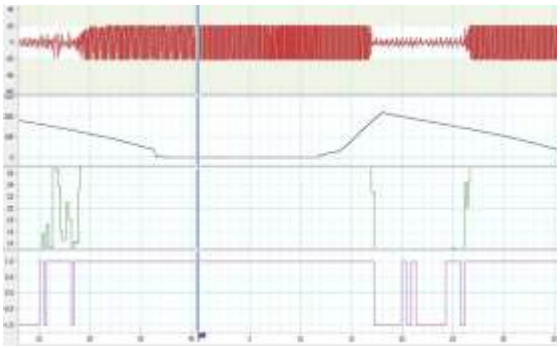

Figure 3: BP in the dulaglutide control group**.**

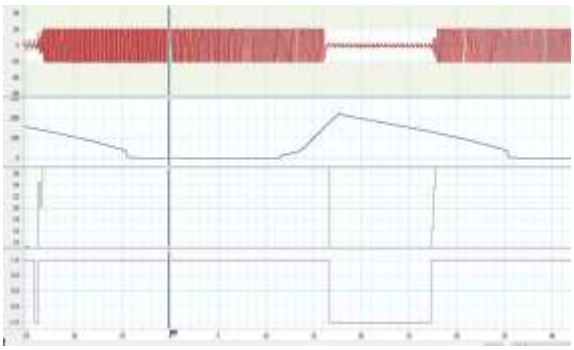

Figure 4: BP in the ivabradine control group

SBP in the dulaglutide- and ivabradine-treated groups was 115.33 ± 3.72 and 125.00 ± 4.47 mmHg respectively (

Table 1, Figure 5, Figure 6, Figure 7, Figure 8).

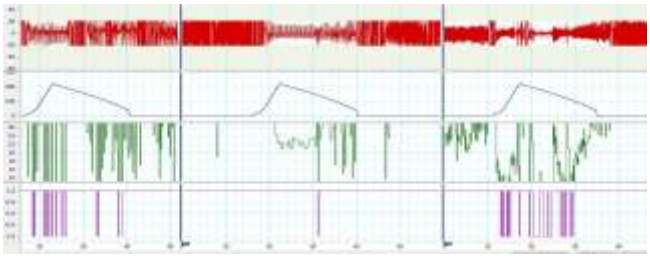

Figure 5: BP in the sucrose group

There was a significant decrease (P < 0.05) in the SBP values between the ivabradine control group and the normal control group but there was an insignificant decrease (P > 0.05) between the dulaglutide control group and the normal control group (

Table 1).

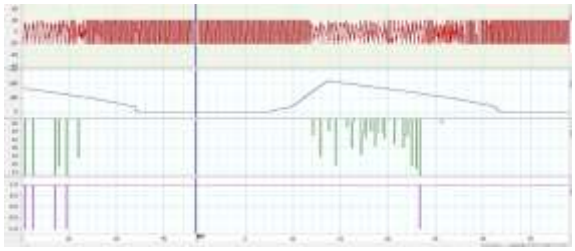

Figure 6: BP in the dulaglutide-treated group

On the other hand, there was a significant increase in SBP (P < 0.05) in the sucrose group compared to the normal control group (

Table 1, Figure 8). There was a significant decrease (P < 0.05) in the dulaglutide- and ivabradine-treated groups compared to the sucrose group (

Table 1).

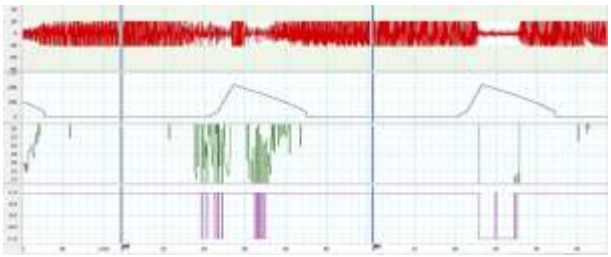

Figure 7: BP in the ivabradine-treated group

2. Diastolic blood pressure (DBP):

The DBP was 89.67 ± 1.36 mmHg in the normal control group. In the dulaglutide and ivabradine control group, DBP was 66.33 ± 4.92 mmHg.
In the sucrose group, DBP was 82.67 ± 8.45 and 78.33 ± 7.60 mmHg (

Table 1, Figure 2, Figure 3, Figure 4). DBP in dulaglutide and ivabradine-treated groups was 73.33 ± 5.164 and 85.00 ± 4.47 mmHg (

Table 1, Figure 5, Figure 6, Figure 7, Figure 8). There was a significant decrease (P < 0.05) in the DBP values between the ivabradine control group and the normal control group.
On the other hand, there was a significant increase in DBP (P < 0.05) in the sucrose group compared to the normal control group (

Table 1). There was a significant decrease in DBP (P < 0.05) in the dulaglutide and ivabradine-treated group compared to the sucrose group (

Table 1).

3. Heart Rate

The heart rate (HR) was 436.67 ± 18.61 beats/ minute in the normal control group. In the dulaglutide and ivabradine control groups, HR was 346.67 ± 20.65 and 240.00 ± 8.94 beats/minute. In the sucrose group, HR was 361.67 ± 29.94 beats/ minute respectively (

Table 1, Figure 2, Figure 3, Figure 4, Figure 9). HR in the dulaglutide and the ivabradine-treated group was 346.67 ± 20.65 and 240.00 ± 8.94 beats/minute respectively (

Table 1, Figure 5, Figure 6, Figure 7, Figure 9).
There was a significant decrease (P < 0.05) in the HR values between all studied groups except for the dulaglutide control and normal control group. There was a significant decrease (P < 0.05) in the HR values between the dulaglutide control and dulaglutide treated groups. Also, a significant decrease (P < 0.05) in the HR values between normal control, ivabradine control and ivabradine treated group compared to the sucrose group (

Table 1).

Table 1: Effect of dulaglutide and ivabradine on blood pressure and heart rate in rats with experimental MS

Groups	SBP (mmHg)	DBP (mmHg)	HR beat/ min
Normal	131.67 ± 2.58	89.67 ± 1.37	436.67 ± 18.62
Dulaglutide	121.33 ± 6.47#	82.67 ± 8.45#	403.33 ± 13.66
Ivabradine	115.0 ± 5.59*#	78.33 ± 7.607#	245.00 ± 4.47*#
Sucrose	166.5 ± 12.33*	110.33 ± 15.60*	361.67 ± 29.94*
Sucrose + Dulaglutide	115.33 ± 3.72*#	73.33 ± 5.16#	346.67 ± 20.66*&
Sucrose + Ivabradine	125.00 ± 4.47#	85.00 ± 4.47#	240.00 ± 8.94*#∞

Each value represents (mean ± standard deviation) (n=6).
* Significant at P < 0.05 compared groups with the normal group.
# Significantly compared groups with the sucrose group.

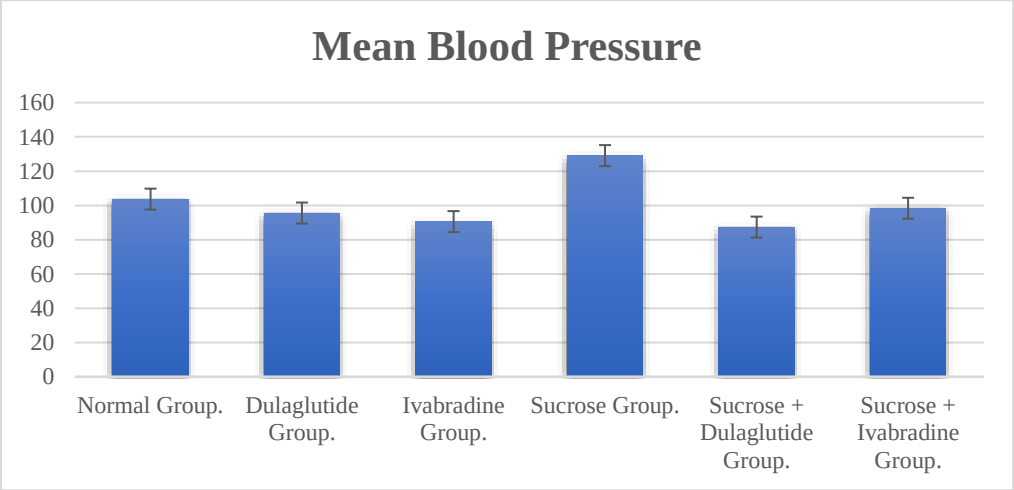

Figure 8: Effect of dulaglutide and ivabradine on systolic blood pressure in male albino rats with sucrose-induced MS

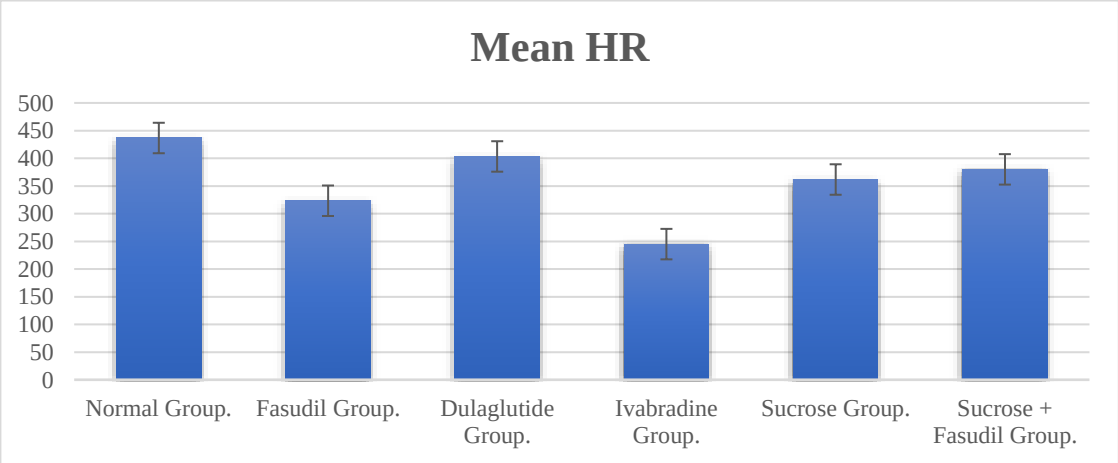

Figure 9: Effect of dulaglutide and ivabradine on heart rate in male albino rats with sucrose-induced MS

B. Body Weight and Biochemistry Parameters

Body Weight

The body weight was 236.67 ± 5.16 gm in the normal control group. In the dulaglutide and ivabradine control group body weight was 170.00 ± 8.94 gm, and 246.67 ± 5.16 gm respectively (Table 2, Figure 10). In the sucrose group, body weight was 266.67 ± 12.11 gm. In the dulaglutide and ivabradine-treated group, it was 203.3 ± 5.16 and 218.3 ± 11.25 respectively (Table 2, Figure 10). There was a significant decrease ($P < 0.05$) in the body weight values in the dulaglutide control and dulaglutide treated groups in comparison to the normal control group.

On the other hand, body weight was significantly increased ($P < 0.05$) in the dulaglutide treated group compared to the dulaglutide control group but there was a significant decrease in body weight ($P < 0.05$) in the ivabradine treated group compared to the ivabradine control group. Also, there was a significant decrease ($P < 0.05$) in the body weight in all studied groups compared to the sucrose group (Table 2).

Serum glucose

The serum glucose was 4.65 ± 0.519 mmol/L in the normal control group. In dulaglutide and ivabradine control groups glucose was 4.895 ± 0.225 and 4.700 ± 0.230 mmol/L respectively (Table 2, Figure 11). In the sucrose group, serum glucose was 20.033 ± 2.27 mmol/L.

Serum glucose in the dulaglutide and ivabradine treated groups was 10.967 ± 2.14 and 10.567 ± 1.73 mmol/L respectively (Table 2, Figure 11). There was a significant decrease ($P < 0.05$) in the serum glucose values between the dulaglutide and ivabradine treated group compared to the sucrose group. Also, there was a significant decrease ($P < 0.05$) in the glucose values in the studied group compared to the sucrose group (Table 2).

Serum insulin

The serum insulin was 8.05 ± 0.29 µIU/L in the normal control group. In dulaglutide and ivabradine control groups insulin was 8.15 ± 0.17 and 7.75 ± 0.17 µIU/L respectively (Table 2, Figure 11). In the sucrose group, serum insulin was 20.93 ± 1.04 µIU/L. Insulin in dulaglutide and ivabradine treated groups was 11.90 ± 2.03 and 12.97 ± 0.69 µIU/L respectively (Table 2, Figure 11).

The insulin values were significantly increased ($P < 0.05$) in sucrose, dulaglutide, and ivabradine treated groups compared to the normal control group. There was a significant increase in insulin ($P < 0.05$) in dulaglutide and ivabradine treated groups compared to dulaglutide and ivabradine control groups. (Table 2).

Glycosylated hemoglobin (Hb A1C)

The HbA1C was 5.20 ± 1.03 mg/dL in the normal control group. In dulaglutide and ivabradine control groups HbA1C was 4.55 ± 0.40 and 4.95 ± 0.29 mg/dL respectively (Table 2, Figure 11). In sucrose group, HbA1C was 8.70 ± 0.498 mg/dL.

HbA1C in dulaglutide and ivabradine treated groups was 6.20 ± 0.17 and 6.05 ± 0.22 mg/dL respectively (Table 2, Figure 11). HbA1C values were significantly increased ($P < 0.05$) in the sucrose group compared to all studied groups. There was a significant increase in HbA1C ($P < 0.05$) in, dulaglutide and ivabradine treated groups compared to dulaglutide and ivabradine control groups (Table 2).

Table 2: Effect of dulaglutide and ivabradine on the body weight, serum glucose, serum insulin, and HbA1C in rats with experimental metabolic syndrome

Groups	Weight (gm)	Glucose (mmol/L)	Insulin (µIU/L)	HbA 1C (mg/dL)
Normal	236.67 ± 5.16	4.65 ± 0.52	8.05 ± 0.29	5.20 ± 1.04
Dulaglutide	170.00 ± 8.94*#	4.9 ± 0.23#	8.15 ± 0.17*#	4.55 ± 0.40#
Ivabradine	246.67 ± 5.16#	4.70 ± 0.23#	7.75 ± 0.17#	4.95 ± 0.29#
Sucrose	266.67 ± 12.11*	20.03 ± 2.28*	20.93 ± 1.05*	8.70 ± 0.45*
Sucrose + Dulaglutide	203.3 ± 5.16*#&	10.967 ± 2.15*#&	11.90 ± 2.03*#&	6.20 ± 0.17*#&
Sucrose + Ivabradine	218.3 ± 11.26*#∞	10.567 ± 1.74*#∞	12.97 ± 0.69*#∞	6.050 ± 0.22#∞

Each value represents (mean ± standard deviation) (n=6).
***** Significant at P < 0.05, compared groups with the normal group.
# Significant, compared groups with the sucrose group.
& Significant, compared dulaglutide treated groups to the dulaglutide control group.
∞ Significant, compared ivabradine treated group to ivabradine control group.

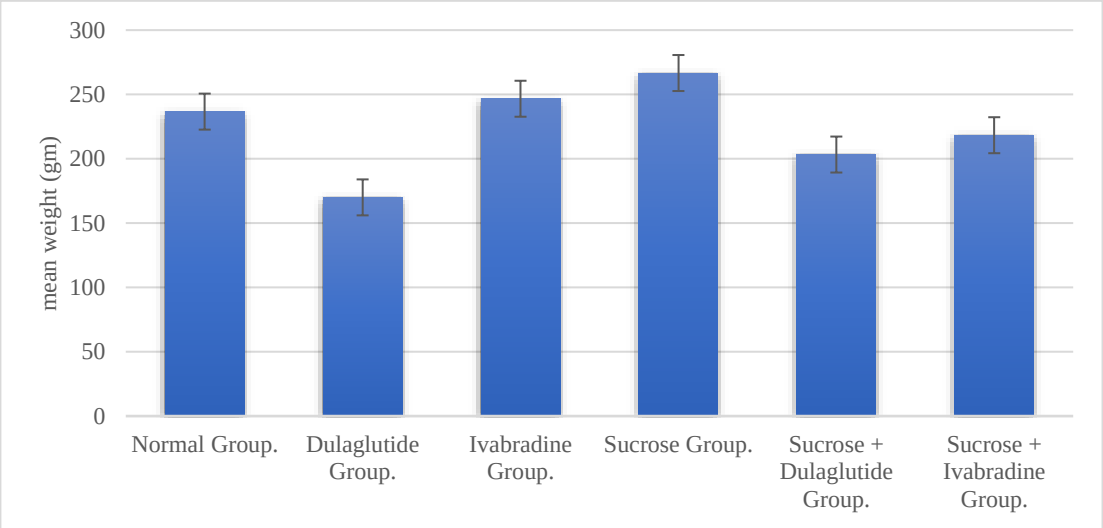

Figure 10: Effect of dulaglutide and ivabradine on body weight in male albino rats with sucrose-induced MS

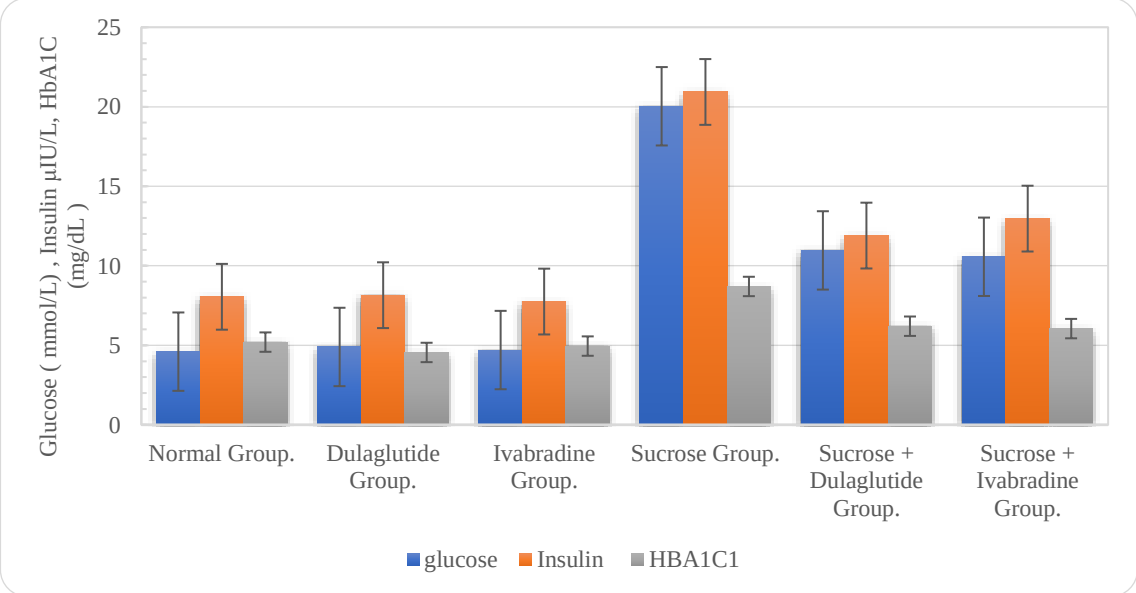

Figure 11: Effect of dulaglutide and ivabradine on Serum Glucose, Insulin, and HbA1C in male albino rats with sucrose-induced MS

Serum LDL

The LDL levels were 131.33 ± 9.39 mg/dl in the normal control group. In dulaglutide and ivabradine control groups, LDL was 128.33 ± 5.82 and 128.33 ± 1.36 mg/dl respectively (Table 3 and Figure13). In sucrose group, LDL was 210.33 ± 9.64 mg/dl.

LDL in dulaglutide and ivabradine treated groups was 146.00 ± 19.41 and 138.33 ± 10.05 mg/dl respectively (Table 3 and Figure 13). The LDL values were significantly increased (P < 0.05) in the sucrose group compared with all studied groups.

There was a significant increase in LDL levels (P < 0.05) in the sucrose group compared to the normal control group. There was a significant increase in LDL levels (P < 0.05) in dulaglutide and ivabradine treated groups compared to control groups.

Serum Nitric oxide (NO)

The NO was 13.83 ± 1.56 nmol/ml in the normal control group. In dulaglutide and ivabradine control groups NO was 14.03 ± 1.56 and 14.367 ± 0.98 nmol/ml respectively (Table 3 and Figure 14). In sucrose group, NO was 89.96 ± 10.64 nmol/ml.

NO in dulaglutide and ivabradine treated groups was 28.400 ± 3.04 and 23.367 ± 3.53 nmol/ml respectively (Table 3 and Figure14). NO values were significantly increased ($P < 0.05$) in sucrose and treated groups compared to the normal control group.

There was a significant increase in NO ($P < 0.05$) in dulaglutide and ivabradine treated groups compared to dulaglutide and ivabradine control groups. Also, there was a significant increase ($P < 0.05$) in the NO in the sucrose group compared with all studied groups.

Cardiac Brain Natriuretic Peptide (BNP)

The BNP was 152.10 ± 8.40 PG/MG protein in the normal control group. In dulaglutide and ivabradine control groups BNP was 157.52 ± 6.88 and 157.73 ± 9.23 PG/MG protein respectively (Table 3 and Figure 15). In sucrose group, BNP was 57.96 ± 5.21 PG/MG protein.

BNP in dulaglutide and ivabradine treated groups was 141.967 ± 10.72 and 139.30 ± 8.63 PG/MG protein respectively (Table 3 and Figure 15). There was a significant decrease ($P < 0.05$) in the BNP values in sucrose compared to the normal control group.

The BNP values were significantly decreased ($P < 0.05$) in dulaglutide and ivabradine treated groups compared to dulaglutide and ivabradine control groups. The BNP values were significantly increased ($P < 0.05$) in dulaglutide and ivabradine treated groups compared to the sucrose group.

Rho kinase enzyme

The Rho levels were 1.90 ± 0.09 ng/ml in the normal control group. In dulaglutide and ivabradine control groups, Rho was 1.80 ± 0.089, 1.87 ± 0.49 ng/ml respectively (Table 3 and figure 16). Rho kinase enzyme in sucrose group was 8.97 ± 1.53 ng/ml, dulaglutide and ivabradine treated groups, Rho was 2.96 ± 0.201 and 2.97 ± 1.04 ng/ml respectively (Table 3 and figure 16).

There was a significant increase ($P < 0.05$) in Rho levels in the sucrose group compared with all studied groups. Also, the "Rho" levels significantly increased ($P < 0.05$) in dulaglutide and ivabradine treated groups compared to normal control groups (Table 3).

Cardiac Proprotein convertase subtilisin/kexin type 9 (PCSK9)

The PCSK9 was 1.005 ± 0.005 pg/mg in the normal control group. In dulaglutide and ivabradine control groups PCSK was 1.02 ± 0.017 and 1.020 ± 0.008 pg/mg respectively (Table 3, Figure 16). In sucrose group, PCSK was 7.40 ± 1.40 pg/mg.

PCSK values in dulaglutide and ivabradine treated groups were 2.83 ± 0.269 and 2.700 ± 0.36 pg/mg respectively (Table 3 and Figure 16). There was a significant increase ($P < 0.05$) in the PCSK levels in sucrose, dulaglutide, and ivabradine treated groups compared to the normal control group.

The PCSK values were significantly increased ($P < 0.05$) in dulaglutide and ivabradine treated groups compared to dulaglutide and ivabradine control groups. The PCSK was significantly increased ($P < 0.05$) in the sucrose group compared to all studied groups.

Table 3: Effect of dulaglutide and ivabradine on serum LDL, NO Rho kinase enzyme, and cardiac BNP and PCSK9 in rats with experimental MS

Groups	LDL (mg/dL)	Rho kinase enzyme (ng/ml)	NO (nmol/ml)	BNP (pg/mg)	PCSK9 (Pg/mg ptn)
Normal.	131.33 ± 9.395	1.903 ± 0.094	13.83 ± 1.56	152.10 ± 8.41	1.005 ± 0.01
Dulaglutide	128.33 ± 5.82 #	1.80 ± 0.089#	14.03 ± 1.56 #	157.52 ± 6.89 #	1.02 ± 0.018 #
Ivabradine	128.33 ± 1.366#	1.867 ± 0.49#	14.37 ± 0.98#	157.73 ± 8.24 #	1.02 ± 0.01 #
Sucrose	210.33 ± 9.65*	8.97 ± 1.54 *	89.96 ± 10.65*	57.96 ± 5.21 *	7.40 ± 1.41*

Sucrose + Dulaglutide	146.0 ± 19.41 #	2.96 ± 0.20#	28.40 ± 3.04 *#&	141.97 ± 10.73 #&	2.83 ± 0.27 *#&
Sucrose + Ivabradine	138.3 ± 10.05 #	2.97 ± 1.05 #	23.37 ± 3.54 *#∞	139.30 ± 8.7#∞	2.70 ± 0.358 *#∞

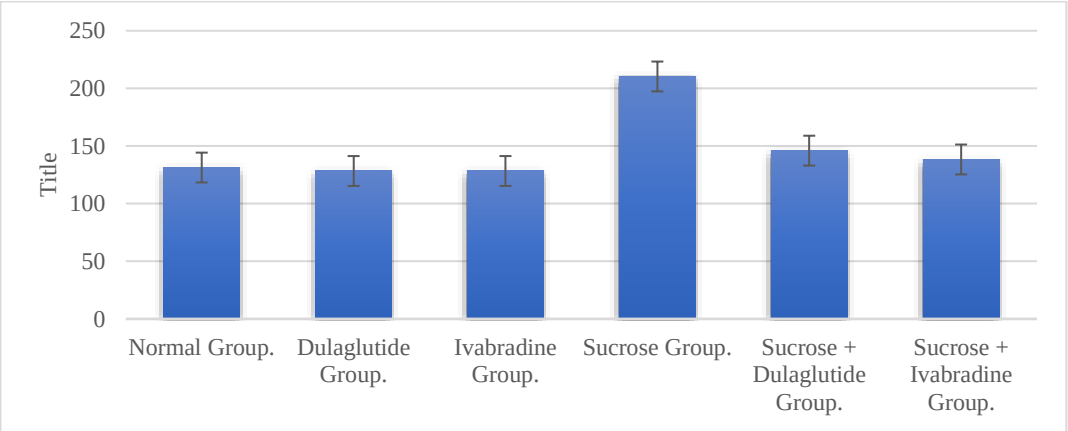

Figure 12: Effect of dulaglutide and ivabradine on serum LDL in male albino rats with sucrose-induced MS.

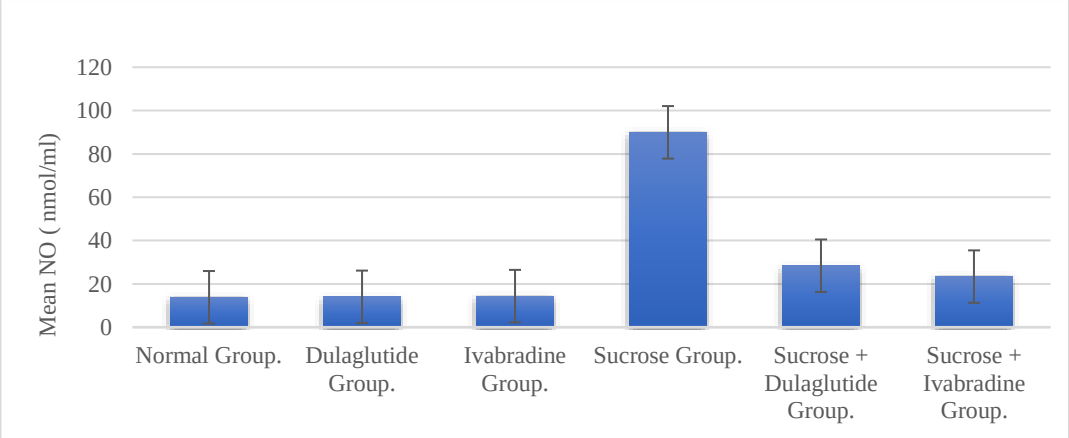

Figure 13: Effect of dulaglutide and ivabradine on serum NO in male albino rats with sucrose-induced MS.

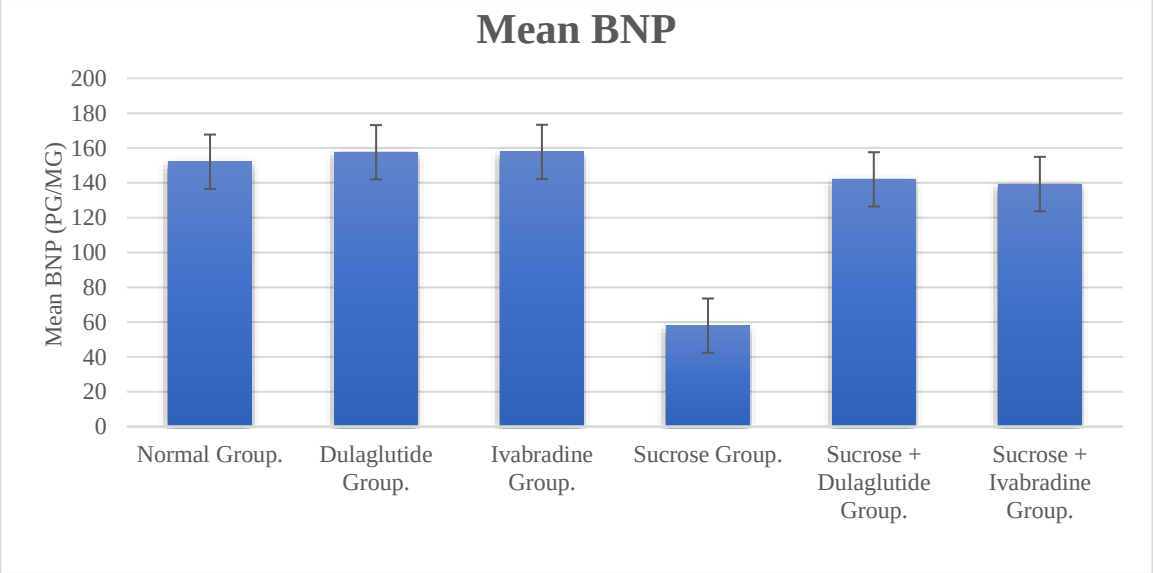

Figure 14: Effect of dulaglutide and ivabradine on BNP in cardiac tissue of male albino rats with sucrose-induced MS

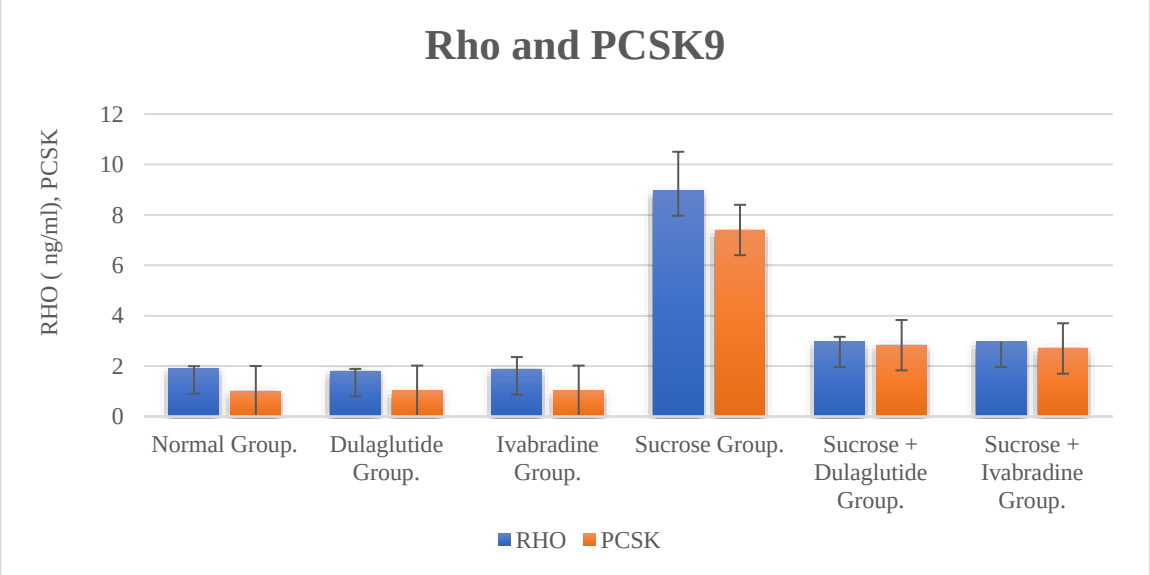

Figure 15: Effect of dulaglutide and ivabradine on Rho and PCSK9 in cardiac tissue of male albino rats with sucrose-induced MS.

Correlation of SBP and (Hb A1C, Rho, NO, BNP and PCSK9)

As shown in Table 4 there was a significant positive correlation between SBP and HbA 1C in the 36 rats (Figure 16). Similarly, there were significant positive correlations between SBP levels and each serum Rho, NO, and PCSK9 (Figure 17, Figure 18, Figure 20). In contrast, there was a significant negative correlation between SBP and BNP (Figure 19)

Table 4: Correlation of SBP and (Hb A1C, Rho, NO, BNP and PCSK9) in male albino rats with sucrose-induced MS.

		HbA1C (mg/dL)	Rho (ng/mL)	NO (nmol/mL)	BNP (PG/MG)	PCSK9 (pg/mg ptn)
Systolic Blood Pressure (mmHg)	**R**	0.609**	0.696**	0.661**	-0.712**	0.603**
	***P* value**	0.000	0.000	0.000	0.000	0.000
	N	48	48	48	48	48

**Correlation is significant at the 0.01 levels (2-tailed). r value: Pearson's Correlation Coefficient, if r value= + .60 or higher Very strong positive relationship and if= -.60 or higher→ Very strong negative relationship. P value was considered significant if < 0.05. N: number of rats.

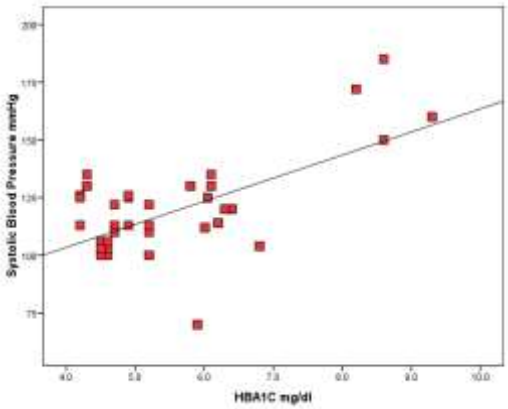

Figure 16: Correlations of serum HbA1C and SBP of male albino rats with sucrose-induced MS.

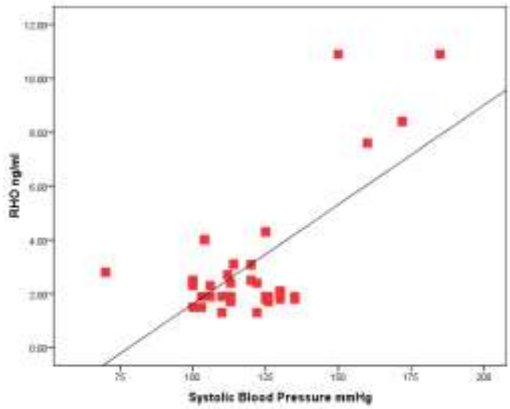

Figure 17: Correlations of SBP and Serum Rho in male albino rats with sucrose-induced MS.

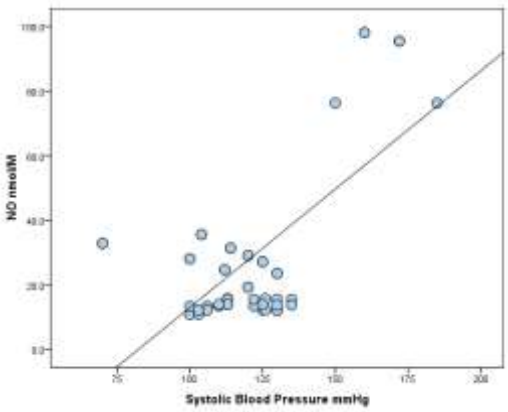

Figure 18: Correlations of SBP and Serum NO in male albino rats with sucrose-induced MS.

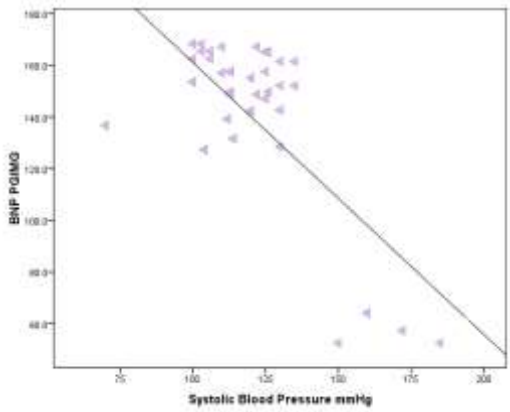

Figure 19: Correlations of SBP and Cardiac BNP of male albino rats with sucrose-induced MS.

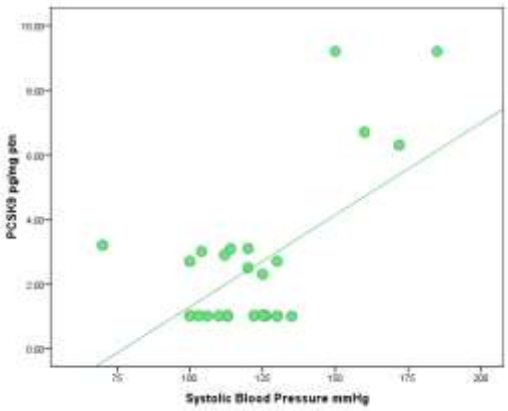

Figure 20: Correlations of SBP and Cardiac PCSK9 of male albino rats with sucrose-induced MS**.**

Correlation of HbA1C and (SBP, Rho, NO, BNP and PCSK9)

As shown in Table 5 there was a significant positive correlation between HbA1C and SBP in the 36 rats (Figure 21). Similarly, there were significant positive correlations between HbA1C levels and serum Rho, NO, and PCSK9 (Figure 25, Figure 22, Figure 24). In contrast, there was a significant negative correlation between HbA1C and BNP (Figure 19).

Table 5: Correlation of HbA1C and (SBP, Rho, NO, BNP and PCSK9) in male albino rats with sucrose-induced MS.

		Systolic Blood Pressure (mmHg)	Rho (ng/mL)	NO (nmol/mL)	BNP (PG/MG)	PCSK9 (pg/mg ptn)
HbA1C (mg/dL)	R	.620**	.864**	.906**	-.870**	.903**
	P value	.000	.000	.000	.000	.000
	N	48	48	48	48	48

**Correlation is significant at the 0.01 levels (2-tailed). r value: Pearson's Correlation Coefficient, if r value= + .60 or higher Very strong positive relationship and if= -.60 or higher→ Very strong negative relationship. P value was considered significant if < 0.05. N: number of rats.

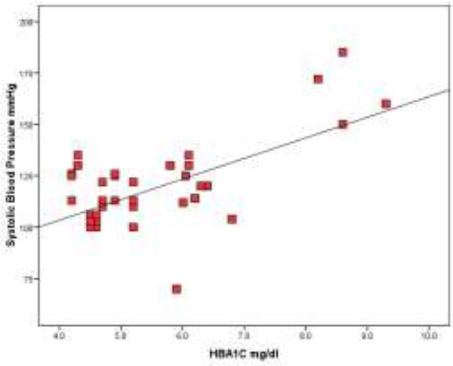

Figure 21: Correlations of serum HbA1C and SBP of male albino rats with sucrose-induced MS

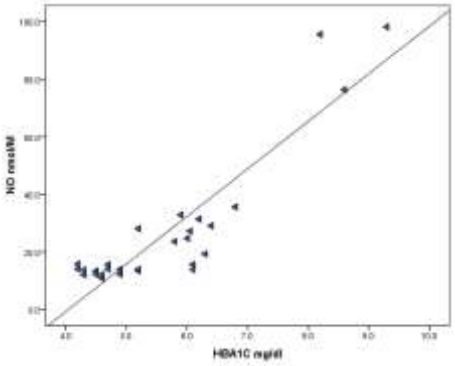

Figure 22: Correlations of serum HbA1C and NO of male albino rats with sucrose-induced MS

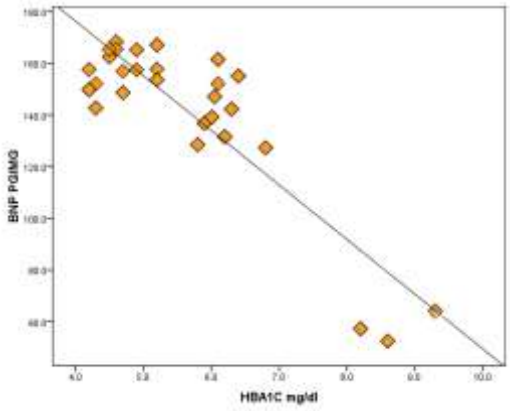

Figure 23: Correlations of serum HbA1C and Cardiac BNP of male albino rats with sucrose-induced MS.

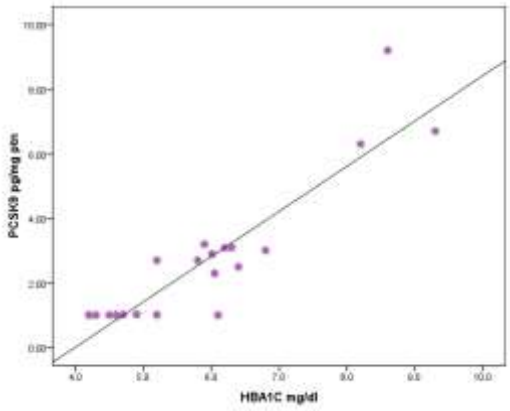

Figure 24: Correlations of serum HbA1C and Cardiac PCSK9 of male albino rats with sucrose-induced MS.

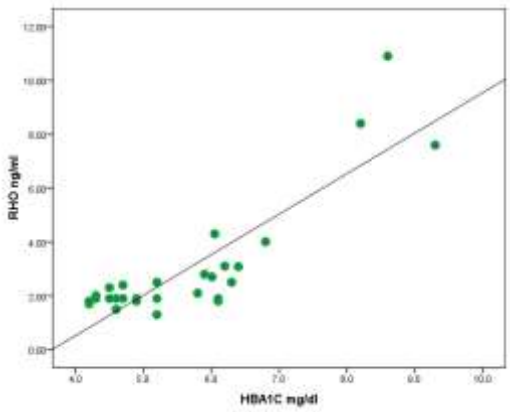

Figure 25: Correlations of serum HbA1C and Rho of male albino rats with Sucrose induced Metabolic Syndrome**.**

Histopathological assessment

In this study, the light microscopic examination by H&E revealed no abnormal microscopy for the cardiac muscles of all four control groups. The cardiac muscle fibers appeared cylindrical, branching with central oval nuclei and acidophilic cytoplasm separated by a minimal amount of connective tissue (Figure 26).

In the sucrose group, myocytes appeared hypertrophied but with no vacuolation of cytoplasm and with mild interstitial fibrosis (Figure 29, Figure 30, Figure 31). Hypertrophy of the myocytes was markedly decreased in all treated groups meanwhile the interstitial fibrosis was markedly decreased in treated groups. Sections stained with Hematoxylin and Eosin were examined and graded for hypertrophy (0 to 3).

All 3 studied control groups recorded grade 0 hypertrophy as there was no abnormality for the cross diameter of myocyte at levels of the nucleus (Table 6, Figure 26, Figure 27, Figure 28). The sucrose group showed the highest histopathological grade (3) for myocyte hypertrophy among all studied groups (Table 6, Figure 29, Figure 30). While, all treated groups showed the lowest histopathological grade (1) for hypertrophy (Table 6, Figure 32, Figure 33, Figure 34, Figure 35). Sections stained by Masson's trichrome were examined and graded for fibrosis. All four studied control groups recorded grade (0) interstitial fibrosis, while the sucrose group showed marked interstitial fibrosis grade (1) (Table 7, Figure 30). The dulaglutide-treated group showed minimal fibrosis (Table 7, Figure 32, Figure 33) while ivabradine treated group showed moderate fibrosis (Table 7, Figure 35).

Table 6: Histopathological grading for myocardial hypertrophy using Hematoxylin and Eosin**.**

		Histopathological grading for myocardial hypertrophy
Controls	Normal	0
	Dulaglutide	0
	Ivabradine	0
Sucrose group		3
Treated groups	Sucrose + Dulaglutide	1
	Sucrose + Ivabradine	1

Table 7: Histopathological grading for myocardial interstitial fibrosis using Masson's trichrome**.**

		Histopathological grading for myocardial interstitial fibrosis
Controls	Normal	0
	Dulaglutide	0
	Ivabradine	0
Sucrose group		1
Treated groups	Sucrose + Dulaglutide	Minimal
	Sucrose + Ivabradine	1

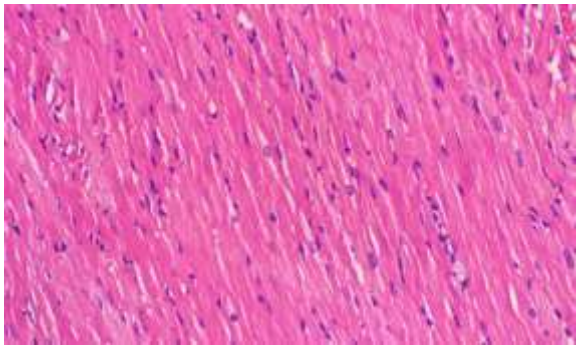

Figure 26: Section in rat's heart muscle of normal control group showing normal thickness and arrangement of cardiac muscle bundles with vesicular central nuclei (H&E × 200).

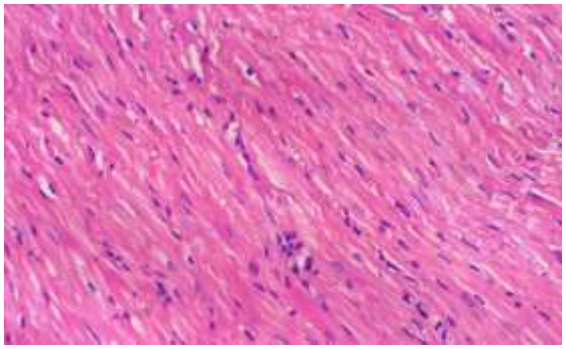

Figure 27: Transverse section in rat's heart muscle of Dulaglutide control group showing normal thickness and arrangement of cardiac muscle bundles with vesicular nuclei (H&E × 200)

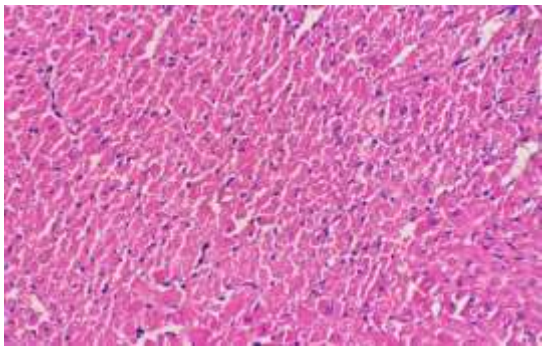

Figure 28: Transverse section in rat's heart muscle of Ivabradine control group showing normal thickness and arrangement of cardiac muscle bundles with central vesicular nuclei with minimal congestion (H&E × 200).

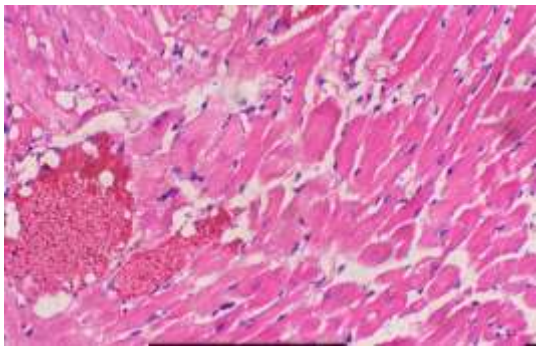

Figure 29: Transverse section in rat's heart muscle of sucrose group showing widely separated cardiac muscles with dark nuclei with marked congestion and vacuolation (H&E × 200).

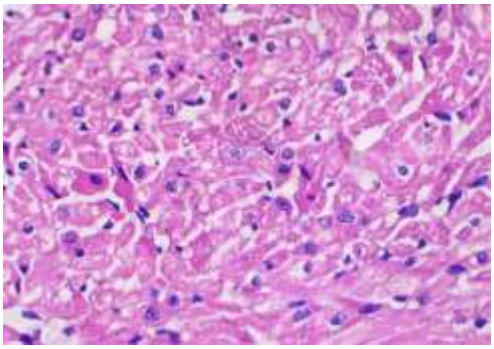

Figure 30: Transverse section in rat's heart muscle of sucrose group showing marked acidophilic cardiac muscles with dark nuclei widely separated cardiac muscles with marked congestion and inflammatory infiltration (H&E × 200).

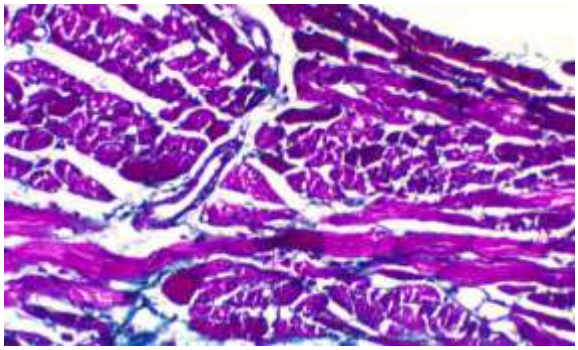

Figure 31: Section in rat's heart muscle of sucrose group showing marked deposition of collagen fiber (Masson's trichrome × 200).

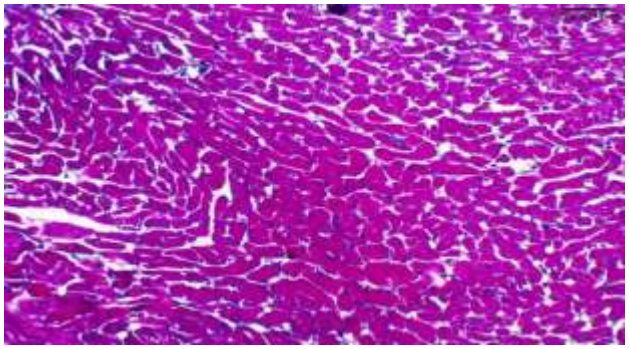

Figure 32: Transverse section in rat's heart muscle of Dulaglutide sucrose group showing minimal widening between layers of cardiac muscle fibers with few lymphocytic infiltrations (H&E × 200).

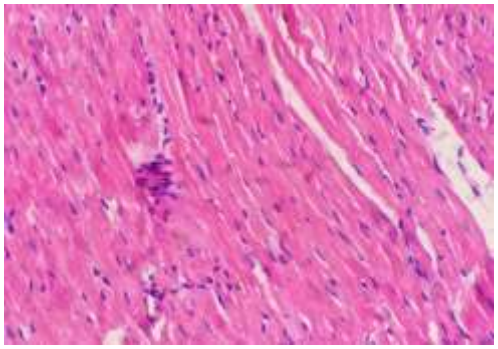

Figure 33: Section in rat's heart muscle of Dulaglutide sucrose treated group showing minimal deposition of collagen fiber (Masson's trichrome × 200).

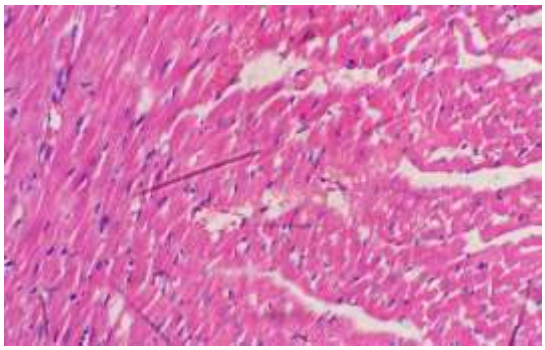

Figure 34: Transverse section in rat's heart muscle of Ivabradine sucrose group showing normal thickness and arrangement of cardiac muscle bundles with vacuolation and congestion (H&E × 200).

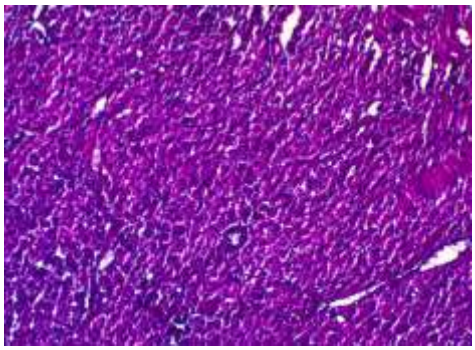

Figure 35: Section in rat's heart muscle of Ivabradine sucrose treated group showing moderate deposition of collagen fiber (Masson's trichrome × 200).

Discussion

The present study demonstrates the effect of dulaglutide (glucagon-like peptide-1 receptor agonist (GLP-1RA) and ivabradine (a hyperpolarization-activated cyclic nucleotide-gated channel blocker that blocks If current, which regulates pacemaker activity on cardiovascular dysfunction in sucrose-induced diabetes in rats).

Metabolic syndrome (MS) represents a clustering of cardiovascular risk factors that include abdominal obesity, dyslipidemia, insulin resistance, and hypertension [52].

Sucrose is a dietary source of fructose. Thus, sucrose feeding has been used to induce metabolic syndrome in animal models. Sucrose-induced lipogenesis in rats with increased plasma concentrations of insulin, leptin, TG, glucose, and FFAs, and impaired glucose tolerance. Consumption of sucrose increases several risk factors for cardiovascular disease by increasing systolic blood pressure in rats with increased left ventricular mass and metabolic syndrome [24].

Dulaglutide is a GLP-1 receptor agonist indicated for improving glycemic control in adults with T2DM adjunct to diet and exercise. Dulaglutide increases insulin secretion when glucose levels are elevated, decreases glucagon secretion, and delays gastric emptying to lower postprandial glucose levels [27]. GLP-1 receptor agonists used to be the first-line treatment in people with T2DM who either are intolerant to metformin or have high CVS risk factors [29].

Ivabradine acts on the If current which is an important ionic current for regulating pacemaker activity in the sino-atrial (SA) node that decreases the heart rate [34].

The sucrose group in the present study presented a significant increase in SBP measured after induction of MS by giving sucrose (30%) in drinking water for 3 weeks.

There was a significant decrease in the SBP and DBP values in all study groups compared to the sucrose group.

Tuttolomondo and colleagues (2021) reported that subjects with T2DM and treated with dulaglutide showed significantly lower SBP and DBP values at the nine-month follow-up [53].

Li et al. (2015) found that ivabradine can improve outcomes in patients with chronic HF and reduce ejection fraction with heart rate >70 bpm despite guidelines-directed medical therapy [27].

Wei et al., (2016) studied ivabradine treatment in L-NAME hypertensive rats for 4 weeks and found that ivabradine reduced systolic blood pressure by 15%, and the 4-week average blood pressure was decreased by 8% compared to that in the diseased group [54]. **Simko et al. (2015)** studied ivabradine's effect on L-NAME-induced hypertension in rats, they found that ivabradine could reduce systolic BP not only in the diseased group (by 21%) but even in the control group (by 26%) [55].

We demonstrated a significant decrease in HR in ivabradine control and ivabradine + sucrose treated groups compared to the sucrose group. This is consistent with the results obtained from Kröller-Schön et al. (2011) who studied the effects of ivabradine in Wistar rats with angiotensin II-induced hypertension, and found that ivabradine sustained 15–20% heart rate reduction [36].

Furthermore, ivabradine added to the established therapy in patients with HR above 70 beats/min significantly reduced the primary combined endpoint of cardiovascular mortality or hospitalization for heart failure [56]. Moreover, ivabradine is of potential benefit in increased heart rate [57] or healthy individuals with supraventricular tachycardia and other off-label uses [58]. Besides its bradycardiac effect, ivabradine exerts several pleiotropic actions such as attenuation of heart remodeling and reduction of fibrosis [55,59,60].

Furthermore, **Krajcirovicova et al. (2018)** concluded that the treatment with ivabradine has consistently reduced the correlation trends between SBP and HR [61].

On contrary, Vaillant et al. (2016) found that ivabradine-treated hearts displayed significantly higher stroke volume values and HR [62].

In our work, we found a significant decrease in the body weight values in treated groups compared to the sucrose group. We also reported a significant weight reduction in the dulaglutide/sucrose-treated group compared to the control group. This agrees with the results obtained from Tuttolomondo et al. (2021) who reported that subjects treated with dulaglutide showed significantly lower mean body weight value at the nine-month follow-up [53]. Numerous clinical trials found that the GLP-1 receptor agonists promote weight loss [63,64].

In all six-phase 3 studies, dulaglutide 1.5 mg resulted in weight reduction. In three of the five Phase 3 studies, dulaglutide 0.75 mg was associated with weight reduction.

Dulaglutide 1.5 mg used as monotherapy, or as an add-on to stable doses of metformin, resulted in weight loss of 2.3–3.0 kg for 6–12 months [65,66]

Jendle et al. (2016), reported that HbA1C decreased in response to dulaglutide 1.5 mg twice weekly [54]. Tuttolomondo et al. (2021) demonstrated that people treated with dulaglutide showed significantly reduced mean FBG and HbA1C levels at the three- and nine-month follow-ups [53].

Our study confirms previous findings concerning the reduction of serum glucose and HbA1C serum levels, e.g., those findings from **Jellinger et al. (2012)** and **Miyagawa et al. (2015)** [67,68].

Grunberger and colleagues (2012) reported a dose-dependent HbA1C reduction for dulaglutide and a greater reduction than placebo [28]. **Miyagawa et al. (2015)** reported that the HbA1C levels were reduced in the dulaglutide group by a mean of -0.61% compared with placebo [68].

The results obtained from **Dungan et al. (2014)** and **Wysham et al. (2014)** support that dulaglutide once weekly can be used as an effective alternative treatment to basal insulin in T2DM patients [69,70]. In addition, dulaglutide significantly increased beta-cell function that was significantly higher with dulaglutide compared with metformin and sitagliptin [71].

There was also a significant decrease in the LDL in all treated groups compared to the sucrose group.

Tuttolomondo et al., (2021) reported that subjects treated with dulaglutide showed significantly lower

mean LDL cholesterol and total cholesterol serum levels at the nine-month follow-up [53].

We found that treatment of rats with dulaglutide and ivabradine significantly decrease levels of nitric oxide. This is consistent with **Ference et al. (2017)** and **Fuechtenbusch et al. (2019)** who showed a beneficial effect of the combination of GLP-1 receptor agonists and insulin on the hyperglycemia-induced oxidative stress and decreased NO that improved endothelial function for patients with T2DM [72,73].

Also, we agreed with **Rohm et al. (2016)** who demonstrated that ivabradine at a dose of (10mg/kg/day) decreased levels of nitric oxide and improved cardiac dysfunction in isoproterenol-induced heart failure in rats [74]. A previous study by **Fuechtenbusch et al. (2019)** demonstrated that the reduction of FBG and improvement in glycemic index are correlated with the reduction of oxidative stress on the vascular endothelium [73].

We found also that treatment of rats with dulaglutide and ivabradine significantly increase levels of (BNP) compared to sucrose rats. Similar results are reported by Alsalame (2020) who investigated the effects of ivabradine on levels of ANP, and BNP and found that levels of BNP were significantly higher among ivabradine-treated rats [75].

We found also that treatment of rats with dulaglutide and ivabradine significantly decrease PCSK9 compared to the sucrose group. These findings are consistent with the results reported by **Meena et al. (2019)** who investigated that treatment with dulaglutide 1.5 mg weakly decreased levels of PCSK9 and LDL in diabetic patients [33].

Nagaike et al. (2019) showed that six-month treatment with dulaglutide improved arterial stiffness, and BNP by reduced PCSK9 in subjects with T2DM [76]. PCSK9 is a liver protease that targets LDL receptors for lysosomal degradation. The therapeutic potential of targeting PCSK9 was validated through Mendelian randomization studies that correlated a deleterious mutation in this gene [77]. A recent study of twelve-month treatment with GLP-1RA, SGLT-2i, and their combination showed greater improvement in vascular markers than insulin treatment in T2DM.

The combined therapy as a second-line therapy was superior to either insulin or GLP-1RA and SGLT-2i separately [78].

In the present work, the histopathological analysis revealed that cardiac tissue of the sucrose group showed widely separated cardiac muscles with dark nuclei marked congestion and vacuolation. Masson's trichrome showed a marked deposition of collagen fibers.

These findings are consistent with previous observations of **Daniels et al. (2012);** however, who found more fibrosis core levels of Masson's trichrome staining in the diabetic cardiac tissue that were associated with depressed mRNA expression of collagens 1 and 3, suggesting which changed rate of collagen degradation rather than enhanced synthesis is responsible for the observed interstitial fibrosis [79].

Cardiomyocyte apoptosis is increased in the process of transition from compensated to decompensated ventricular dysfunction in the diabetic heart [80], as dead cardiac myocytes are replaced by extracellular matrix components, which leads to the development of collagen deposition and fibrosis [81,82].

Thent et al. (2012) concluded that inflammatory histological changes in cardiac tissues indicated myocardial injury [83]. Deformation of nuclei of cardiomyocytes and disarrangement or disordered cardiac myofibers was observed under H&E staining. With Masson's trichrome staining, connective tissue deposits were observed.

These findings were considered important for the development of diabetic cardiomyopathy. Previous studies reported diabetic cardiomyopathy characterized by systolic or diastolic dysfunction and cardiac fibrosis in diabetic patients [84].

Thus, several lines of evidence (e.g., **Gerstein et al., 2019; Nagaike et al., 2019; Ikonomidis et al., 2020**) have reported that antidiabetic drugs differentially affect endothelial function and arterial stiffness [76,78,85].

In current work, cardiac tissue of dulaglutide-diabetic rats showed minimal widening between layers of cardiac muscle fibers with few lymphocytic infiltrations. In this study, in ivabradine-treated groups, cardiac tissue showed

normal thickness and arrangement of cardiac muscle bundles with vacuolation and congestion which is consistent with **Khaleel et al. (2019)** who reported that ivabradine showed vascular congestions between myocardial fibers by increasing spaces between myocardial fibers, cardiac atrophy and myocardial degeneration in the heart [86].

Conclusion

Our study proved that both dulaglutide and ivabradine have protective cardiac effect in sucrose-induced metabolic syndrome mainly through amelioration of systolic blood pressure, glycemic indices, serum LDL, serum Rho kinase, serum NO, and (BNP and PCSK9) levels in cardiac tissues.

The serum levels of glucose, insulin, and HbA1C were decreased with dulaglutide and ivabradine treated groups compared with the sucrose group. Similarly, the serum levels of LDL, NO, and Rho were decreased with dulaglutide, and ivabradine treated groups compared with the sucrose group.

PCSK9 levels significantly decreased in cardiac tissues of sucrose groups taking either of the two drugs as compared to the untreated sucrose group. BNP levels were increased with the two drugs. There was a significant increase in SBP in the sucrose group and a significant decrease in dulaglutide- and ivabradine-treated groups. With the sucrose group, there were degenerative changes in cardiac tissue which were less with the three drugs.

In conclusion, both dulaglutide and ivabradine modulate most degenerative changes caused by metabolic syndrome which was induced by sucrose-feeding in drinking water.

RECOMMENDATIONS

Further experimental studies on a larger number of animals and for longer periods are required to study the safety of drugs on other body organs. In addition, research is necessary to explain the molecular mechanism through which dulaglutide and ivabradine produce the cardioprotective effect.

Further clinical studies are required to confirm the experimental data and elucidate the potential protective role of dulaglutide and ivabradine on cardiac complications of metabolic syndrome like vasoreactivity studies, and other cardiac investigations such as ECG and ECHO cardiography and renal function tests.

REFERENCES

1. Tran, L.T., MacLeod, K.M., and McNeill, J.H. (2014) Selective alpha1-adrenoceptor blockade prevents fructose-induced hypertension. *Mol. Cell. Biochem.*, **392** (1–2), 205–211.
2. Hooijschuur, M.C.E., Ghossein-Doha, C., Kroon, A.A., De Leeuw, P.W., Zandbergen, A.A.M., Van Kuijk, S.M.J., and Spaanderman, M.E.A. (2019) Metabolic syndrome and pre-eclampsia. *Ultrasound Obstet. Gynecol.*, **54** (1), 64–71.
3. Levy, J.M. (2020) Endogenous cannabinoids may regulate chronic inflammation in aspirin-exacerbated respiratory disease. *World J. Otorhinolaryngol. - head neck Surg.*, **6** (4), 255–257.
4. Whitney, E.N., and Rolfes, S.R. (2019) *Understanding Nutrition - Cengage*, Stamford, CT : Cengage Learning, Belmont.
5. McCracken, E., Monaghan, M., and Sreenivasan, S. (2018) Pathophysiology of the metabolic syndrome. *Clin. Dermatol.*, **36** (1), 14–20.
6. van Bodegom, M., Homberg, J.R., and Henckens, M.J.A.G. (2017) Modulation of the Hypothalamic-Pituitary-Adrenal Axis by Early Life Stress Exposure. *Front. Cell. Neurosci.*, **11**, 87.
7. Wang, H.H., Lee, D.K., Liu, M., Portincasa, P., and Wang, D.Q.-H. (2020) Novel Insights into the Pathogenesis and Management of the Metabolic Syndrome. *Pediatr. Gastroenterol. Hepatol. Nutr.*, **23** (3), 189–230.
8. Loscalzo, J., Fauci, A.S., Kasper, D.L., Hauser, S., Longo, D., and Jameson, J.L. (2015) *Harrison's Principles of Internal Medicine*, New York : McGraw Hill Education Medical, Washington, DC.
9. Akaberi, M., and Hosseinzadeh, H. (2016) Grapes (Vitis vinifera) as a Potential Candidate for the Therapy of the Metabolic Syndrome. *Phytother. Res.*, **30** (4), 540–56.
10. Frühbeck, G., Catalán, V., Rodríguez, A., Ramírez, B., Becerril, S., Salvador, J., Portincasa, P., Colina, I., and Gómez-Ambrosi, J. (2017) Involvement of the leptin-adiponectin axis in inflammation and oxidative stress in the metabolic syndrome. *Sci. Rep.*, **7** (1), 6619.
11. Cooke, A.A., Connaughton, R.M., Lyons, C.L., McMorrow, A.M., and Roche, H.M. (2016) Fatty acids and chronic low grade inflammation associated with obesity and the metabolic syndrome. *Eur. J. Pharmacol.*, **785**, 207–214.
12. Mohallem, R., and Aryal, U.K. (2020) Regulators of TNFα mediated insulin resistance elucidated by quantitative proteomics. *Sci. Rep.*, **10** (1), 20878.

13. Stefani, G.P., Baldissera, G., Nunes, R.B., Heck, T.G., and Rhoden, C.R. (2015) Metabolic Syndrome and DNA Damage: The Interplay of Environmental and Lifestyle Factors in the Development of Metabolic Dysfunction. *Open J. Endocr. Metab. Dis.*, **05** (07), 65–76.
14. Kensara, O.A. (2018) Prevalence of hypovitaminosis D, and its association with hypoadiponectinemia and hyperfollistatinemia, in Saudi women with naïve polycystic ovary syndrome. *J. Clin. Transl. Endocrinol.*, **12**, 20–25.
15. Lopaschuk, G. Metabolic modulators in heart disease: past, present, and future.". *Can J Cardiol*, **33** (7), 838–849.
16. Brown, S.M., Meuth, A.I., Davis, J.W., Rector, R.S., and Bender, S.B. (2018) Mineralocorticoid receptor antagonism reverses diabetes-related coronary vasodilator dysfunction: A unique vascular transcriptomic signature. *Pharmacol. Res.*, **134**, 100–108.
17. Lamster, I.B., and Pagan, M. (2017) Periodontal disease and the metabolic syndrome. *Int. Dent. J.*, **67** (2), 67–77.
18. Nguyen, K.A., Peer, N., Mills, E.J., and Kengne, A.P. (2016) A Meta-Analysis of the Metabolic Syndrome Prevalence in the Global HIV-Infected Population. *PLoS One*, **11** (3), e0150970.
19. Gaines, J., Vgontzas, A.N., Fernandez-Mendoza, J., and Bixler, E.O. (2018) Obstructive sleep apnea and the metabolic syndrome: The road to clinically-meaningful phenotyping, improved prognosis, and personalized treatment. *Sleep Med. Rev.*, **42**, 211–219.
20. Koren, D., Dumin, M., and Gozal, D. (2016) Role of sleep quality in the metabolic syndrome. *Diabetes. Metab. Syndr. Obes.*, **9**, 281–310.
21. Ussar, S., Griffin, N.W., Bezy, O., Fujisaka, S., Vienberg, S., Softic, S., Deng, L., Bry, L., Gordon, J.I., and Kahn, C.R. (2015) Interactions between Gut Microbiota, Host Genetics and Diet Modulate the Predisposition to Obesity and Metabolic Syndrome. *Cell Metab.*, **22** (3), 516–530.
22. Rippe, J.M., Sievenpiper, J.L., Lê, K.-A., White, J.S., Clemens, R., and Angelopoulos, T.J. (2017) What is the appropriate upper limit for added sugars consumption? *Nutr. Rev.*, **75** (1), 18–36.
23. Stanhope, K.L., Schwarz, J.-M., and Havel, P.J. (2013) Adverse metabolic effects of dietary fructose: results from the recent epidemiological, clinical, and mechanistic studies. *Curr. Opin. Lipidol.*, **24** (3), 198–206.
24. Esser, N., Paquot, N., and Scheen, A.J. (2015) Anti-inflammatory agents to treat or prevent type 2 diabetes, metabolic syndrome and cardiovascular disease. *Expert Opin. Investig. Drugs*, **24** (3), 283–307.
25. Iqbal, J., Al Qarni, A., Hawwari, A., Alghanem, A.F., and Ahmed, G. (2018) Metabolic Syndrome, Dyslipidemia and Regulation of Lipoprotein Metabolism. *Curr. Diabetes Rev.*, **14** (5), 427–433.
26. Alwahsh, S.M., and Gebhardt, R. (2017) Dietary fructose as a risk factor for non-alcoholic fatty liver disease (NAFLD). *Arch. Toxicol.*, **91** (4), 1545–1563.
27. Usborne, A., Byrd, R.A., Meehan, J., Blackbourne, J.L., Sullivan, J., Poitout-Belissent, F., Prefontaine, A., Martin, J.A., and Vahle, J.L. (2015) An Investigative Study of Pancreatic Exocrine Biomarkers, Histology, and Histomorphometry in Male Zucker Diabetic Fatty (ZDF) Rats Given Dulaglutide by Subcutaneous Injection Twice Weekly for 13 Weeks. *Toxicol. Pathol.*, **43** (8), 1093–102.
28. Grunberger, G., Chang, A., Garcia Soria, G., Botros, F.T., Bsharat, R., and Milicevic, Z. (2012) Monotherapy with the once-weekly GLP-1 analogue dulaglutide for 12 weeks in patients with Type 2 diabetes: dose-dependent effects on glycaemic control in a randomized, double-blind, placebo-controlled study. *Diabet. Med.*, **29** (10), 1260–7.
29. Bahtiyar, G., Pujals-Kury, J., and Sacerdote, A. (2018) Cardiovascular Effects of Different GLP-1 Receptor Agonists in Patients with Type 2 Diabetes. *Curr. Diab. Rep.*, **18** (10), 92.
30. Ji, Y., Zhao, Z., Cai, T., Yang, P., and Cheng, M. (2014) Liraglutide alleviates diabetic cardiomyopathy by blocking CHOP-triggered apoptosis via the inhibition of the IRE-α pathway. *Mol. Med. Rep.*, **9** (4), 1254–8.
31. Noyan-Ashraf, M.H., Shikatani, E.A., Schuiki, I., Mukovozov, I., Wu, J., Li, R.-K., Volchuk, A., Robinson, L.A., Billia, F., Drucker, D.J., and Husain, M. (2013) A glucagon-like peptide-1 analog reverses the molecular pathology and cardiac dysfunction of a mouse model of obesity. *Circulation*, **127** (1), 74–85.
32. Monji, A., Mitsui, T., Bando, Y.K., Aoyama, M., Shigeta, T., and Murohara, T. (2013) Glucagon-like peptide-1 receptor activation reverses cardiac remodeling via normalizing cardiac steatosis and oxidative stress in type 2 diabetes. *Am. J. Physiol. Heart Circ. Physiol.*, **305** (3), H295-304.
33. Jain, M., Carlson, G., Cook, W., Morrow, L., Petrone, M., White, N.E., Wang, T., Naylor, J., Ambery, P., Lee, C., and Hirshberg, B. (2019) Randomised, phase 1, dose-finding study of MEDI4166, a PCSK9 antibody and GLP-1 analogue fusion molecule, in overweight or obese patients with type 2 diabetes mellitus. *Diabetologia*, **62** (3), 373–386.
34. Roger, V.L. (2013) Epidemiology of heart failure. *Circ. Res.*, **113** (6), 646–59.
35. Li, S.J., Sartipy, U., Lund, L.H., Dahlstrom, U., Adiels, M., Petzold, M., and Fu, M. (2015)

Prognostic Significance of Resting Heart Rate and Use of β-Blockers in Atrial Fibrillation and Sinus Rhythm in Patients with Heart Failure and Reduced Ejection Fraction : Findings from the Swedish Heart Failure Registry. *Circ. Hear. Fail.*, **8** (5), 871–879.

36. Kröller-Schön, S., Schulz, E., Wenzel, P., Kleschyov, A.L., Hortmann, M., Torzewski, M., Oelze, M., Renné, T., Daiber, A., and Münzel, T. (2011) Differential effects of heart rate reduction with ivabradine in two models of endothelial dysfunction and oxidative stress. *Basic Res. Cardiol.*, **106** (6), 1147–58.
37. Ruwald, M.H., Goetze, J.P., Bech, J., Nielsen, O.W., Madsen, B.K., Nielsen, L.B., Mouridsen, M., Ruwald, A.-C.H., Madsen, J.K., and Pedersen, S. (2014) NT-ProBNP independently predicts long-term mortality in patients admitted for coronary angiography. *Angiology*, **65** (1), 31–6.
38. Matheus, A.S. de M., Tannus, L.R.M., Cobas, R.A., Palma, C.C.S., Negrato, C.A., and Gomes, M. de B. (2013) Impact of diabetes on cardiovascular disease: an update. *Int. J. Hypertens.*, **2013**, 653789.
39. Emdin, C.A., Rahimi, K., Neal, B., Callender, T., Perkovic, V., and Patel, A. (2015) Blood pressure lowering in type 2 diabetes: a systematic review and meta-analysis. *JAMA*, **313** (6), 603–15.
40. Adel, H., Taye, A., and Khalifa, M.M.A. (2014) Spironolactone improves endothelial dysfunction in streptozotocin-induced diabetic rats. *Naunyn. Schmiedebergs. Arch. Pharmacol.*, **387** (12), 1187–1197.
41. Wu, Q., Tang, Z.-H., Peng, J., Liao, L., Pan, L.-H., Wu, C.-Y., Jiang, Z.-S., Wang, G.-X., and Liu, L.-S. (2014) The dual behavior of PCSK9 in the regulation of apoptosis is crucial in Alzheimer's disease progression (Review). *Biomed. reports*, **2** (2), 167–171.
42. Sabatine, M.S., Leiter, L.A., Wiviott, S.D., Giugliano, R.P., Deedwania, P., De Ferrari, G.M., Murphy, S.A., Kuder, J.F., Gouni-Berthold, I., Lewis, B.S., Handelsman, Y., Pineda, A.L., Honarpour, N., Keech, A.C., Sever, P.S., and Pedersen, T.R. (2017) Cardiovascular safety and efficacy of the PCSK9 inhibitor evolocumab in patients with and without diabetes and the effect of evolocumab on glycaemia and risk of new-onset diabetes: a prespecified analysis of the FOURIER randomised controlled trial. *lancet. Diabetes Endocrinol.*, **5** (12), 941–950.
43. Celestine, O.A., Adugba, O.A., Nworgu, C.C., Uzoigwe, U.J., Okorie, O.P., Agu, U.F., Ediale, R.J., Egharevba, E.J., and Nwachukwu, C.D. (2017) Investigation of antihypertensive effect of Nigerian varieties of Solanum lycopersicon on rats. *African J. Pharm. Pharmacol.*, **11** (34), 419–425.
44. Barham, D., and Trinder, P. (1972) An improved colour reagent for the determination of blood glucose by the oxidase system. *Analyst*, **97** (151), 142–5.
45. Peng, G., Lin, M., Zhang, K., Chen, J., Wang, Y., Yang, Y., Wang, J., and Huang, H. (2013) Hemoglobin A1c can identify more cardiovascular and metabolic risk profile in OGTT-negative Chinese population. *Int. J. Med. Sci.*, **10** (8), 1028–1034.
46. Miller, W.G., Myers, G.L., Sakurabayashi, I., Bachmann, L.M., Caudill, S.P., Dziekonski, A., Edwards, S., Kimberly, M.M., Korzun, W.J., Leary, E.T., Nakajima, K., Nakamura, M., Nilsson, G., Shamburek, R.D., Vetrovec, G.W., Warnick, G.R., and Remaley, A.T. (2010) Seven direct methods for measuring HDL and LDL cholesterol compared with ultracentrifugation reference measurement procedures. *Clin. Chem.*, **56** (6), 977–86.
47. Kleschyov, A.L., Wenzel, P., and Munzel, T. (2007) Electron paramagnetic resonance (EPR) spin trapping of biological nitric oxide. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, **851** (1–2), 12–20.
48. Judzewitsch, R.G., Pfeifer, M.A., Best, J.D., Beard, J.C., Halter, J.B., and Porte, D. (1982) Chronic chlorpropamide therapy of noninsulin-dependent diabetes augments basal and stimulated insulin secretion by increasing islet sensitivity to glucose. *J. Clin. Endocrinol. Metab.*, **55** (2), 321–8.
49. Jeong, K.J., Park, S.Y., Cho, K.H., Sohn, J.S., Lee, J., Kim, Y.K., Kang, J., Park, C.G., Han, J.W., and Lee, H.Y. (2012) The Rho/ROCK pathway for lysophosphatidic acid-induced proteolytic enzyme expression and ovarian cancer cell invasion. *Oncogene*, **31** (39), 4279–4289.
50. Cowie, M.R., Jourdain, P., Maisel, A., Dahlstrom, U., Follath, F., Isnard, R., Luchner, A., McDonagh, T., Mair, J., Nieminen, M., and Francis, G. (2003) Clinical applications of B-type natriuretic peptide (BNP) testing. *Eur. Heart J.*, **24** (19), 1710–8.
51. Sucajtys-Szulc, E., Szolkiewicz, M., Swierczynski, J., and Rutkowski, B. (2016) Up-regulation of Hnf1α gene expression in the liver of rats with experimentally induced chronic renal failure – A possible link between circulating PCSK9 and triacylglycerol concentrations. *Atherosclerosis*, **248**, 17–26.
52. Amisten, S., Salehi, A., Rorsman, P., Jones, P.M., and Persaud, S.J. (2013) An atlas and functional analysis of G-protein coupled receptors in human islets of Langerhans. *Pharmacol. Ther.*, **139** (3), 359–91.
53. Tuttolomondo, A., Cirrincione, A., Casuccio, A., Del Cuore, A., Daidone, M., Di Chiara, T., Di

Raimondo, D., Corte, V. Della, Maida, C., Simonetta, I., Scaglione, S., and Pinto, A. (2021) Efficacy of dulaglutide on vascular health indexes in subjects with type 2 diabetes: a randomized trial. *Cardiovasc. Diabetol.*, **20** (1), 1.

54. Jendle, J., Grunberger, G., Blevins, T., Giorgino, F., Hietpas, R.T., and Botros, F.T. (2016) Efficacy and safety of dulaglutide in the treatment of type 2 diabetes: a comprehensive review of the dulaglutide clinical data focusing on the AWARD phase 3 clinical trial program. *Diabetes. Metab. Res. Rev.*, **32** (8), 776–790.
55. Simko, F., Repova, K., Krajcirovicova, K., Aziriova, S., Paulis, L., and Baka, T. (2015) Remodelling of the aorta and kidney in L-NAME-induced hypertension in rats: comparison of the protective effect of ivabradine with captopril and melatonin. *Diabetologia*, **58**.
56. Swedberg, K., Komajda, M., Böhm, M., Borer, J.S., Ford, I., Dubost-Brama, A., Lerebours, G., Tavazzi, L., and SHIFT Investigators (2010) Ivabradine and outcomes in chronic heart failure (SHIFT): a randomised placebo-controlled study. *Lancet (London, England)*, **376** (9744), 875–85.
57. Palatini, P., Rosei, E.A., Casiglia, E., Chalmers, J., Ferrari, R., Grassi, G., Inoue, T., Jelakovic, B., Jensen, M.T., Julius, S., Kjeldsen, S.E., Mancia, G., Parati, G., Pauletto, P., Stella, A., and Zanchetti, A. (2016) Management of the hypertensive patient with elevated heart rate: Statement of the Second Consensus Conference endorsed by the European Society of Hypertension. *J. Hypertens.*, **34** (5), 813–21.
58. Oliphant, C.S., Owens, R.E., Bolorunduro, O.B., and Jha, S.K. (2016) Ivabradine: A Review of Labeled and Off-Label Uses. *Am. J. Cardiovasc. Drugs*, **16** (5), 337–347.
59. Mulder, P., Barbier, S., Chagraoui, A., Richard, V., Henry, J.P., Lallemand, F., Renet, S., Lerebours, G., Mahlberg-Gaudin, F., and Thuillez, C. (2004) Long-term heart rate reduction induced by the selective I(f) current inhibitor ivabradine improves left ventricular function and intrinsic myocardial structure in congestive heart failure. *Circulation*, **109** (13), 1674–9.
60. Kleinbongard, P., Gedik, N., Witting, P., Freedman, B., Klöcker, N., and Heusch, G. (2015) Pleiotropic, heart rate-independent cardioprotection by ivabradine. *Br. J. Pharmacol.*, **172** (17), 4380–90.
61. Krajcirovicova, K., Aziriova, S., Baka, T., Repova, K., Adamcova, M., Paulis, L., and Simko, F. (2018) Ivabradine does not impair anxiety-like behavior and memory in both healthy and L-NAME-induced hypertensive rats. *Physiol. Res.*, **67** (Suppl 4), S655–S664.
62. Vaillant, F., Lauzier, B., Ruiz, M., Shi, Y., Lachance, D., Rivard, M.-E., Bolduc, V., Thorin, E., Tardif, J.-C., and Des Rosiers, C. (2016) Ivabradine and metoprolol differentially affect cardiac glucose metabolism despite similar heart rate reduction in a mouse model of dyslipidemia. *Am. J. Physiol. Heart Circ. Physiol.*, **311** (4), H991–H1003.
63. Mann, K. V, and Raskin, P. (2014) Exenatide extended-release: a once weekly treatment for patients with type 2 diabetes. *Diabetes. Metab. Syndr. Obes.*, **7**, 229–39.
64. Rigato, M., and Fadini, G.P. (2014) Comparative effectiveness of liraglutide in the treatment of type 2 diabetes. *Diabetes. Metab. Syndr. Obes.*, **7**, 107–20.
65. Garber, A., Henry, R., Ratner, R., Garcia-Hernandez, P.A., Rodriguez-Pattzi, H., Olvera-Alvarez, I., Hale, P.M., Zdravkovic, M., Bode, B., and LEAD-3 (Mono) Study Group (2009) Liraglutide versus glimepiride monotherapy for type 2 diabetes (LEAD-3 Mono): a randomised, 52-week, phase III, double-blind, parallel-treatment trial. *Lancet (London, England)*, **373** (9662), 473–81.
66. Diamant, M., Van Gaal, L., Stranks, S., Northrup, J., Cao, D., Taylor, K., and Trautmann, M. (2010) Once weekly exenatide compared with insulin glargine titrated to target in patients with type 2 diabetes (DURATION-3): an open-label randomised trial. *Lancet (London, England)*, **375** (9733), 2234–43.
67. Jellinger, P.S., Smith, D.A., Mehta, A.E., Ganda, O., Handelsman, Y., Rodbard, H.W., Shepherd, M.D., and Seibel, J.A. (2012) American Association of Clinical Endocrinologists' Guidelines for Management of Dyslipidemia and Prevention of Atherosclerosis. *Endocr. Pract.*, **18**, 1–78.
68. Miyagawa, J., Odawara, M., Takamura, T., Iwamoto, N., Takita, Y., and Imaoka, T. (2015) Once-weekly glucagon-like peptide-1 receptor agonist dulaglutide is non-inferior to once-daily liraglutide and superior to placebo in Japanese patients with type 2 diabetes: a 26-week randomized phase III study. *Diabetes. Obes. Metab.*, **17** (10), 974–83.
69. Dungan, K.M., Povedano, S.T., Forst, T., González, J.G.G., Atisso, C., Sealls, W., and Fahrbach, J.L. (2014) Once-weekly dulaglutide versus once-daily liraglutide in metformin-treated patients with type 2 diabetes (AWARD-6): a randomised, open-label, phase 3, non-inferiority trial. *Lancet (London, England)*, **384** (9951), 1349–57.
70. Wysham, C., Blevins, T., Arakaki, R., Colon, G., Garcia, P., Atisso, C., Kuhstoss, D., and Lakshmanan, M. (2014) Efficacy and Safety of Dulaglutide Added Onto Pioglitazone and Metformin Versus Exenatide in Type 2 Diabetes in a Randomized Controlled Trial (AWARD-1).

Diabetes Care, **37** (8), 2159–2167.
71. Umpierrez, G., Tofé Povedano, S., Pérez Manghi, F., Shurzinske, L., and Pechtner, V. (2014) Efficacy and safety of dulaglutide monotherapy versus metformin in type 2 diabetes in a randomized controlled trial (AWARD-3). *Diabetes Care*, **37** (8), 2168–76.
72. Ference, B.A., Ginsberg, H.N., Graham, I., Ray, K.K., Packard, C.J., Bruckert, E., Hegele, R.A., Krauss, R.M., Raal, F.J., Schunkert, H., Watts, G.F., Borén, J., Fazio, S., Horton, J.D., Masana, L., Nicholls, S.J., Nordestgaard, B.G., van de Sluis, B., Taskinen, M.-R., Tokgözoglu, L., Landmesser, U., Laufs, U., Wiklund, O., Stock, J.K., Chapman, M.J., and Catapano, A.L. (2017) Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. *Eur. Heart J.*, **38** (32), 2459–2472.
73. Fuechtenbusch, M., Aberle, J., Heitmann, E., Nicolay, C., and Jung, H. (2019) Weight loss in patients with type 2 diabetes receiving once-weekly dulaglutide plus insulin lispro or insulin glargine plus insulin lispro: A post-hoc analysis of the AWARD-4 study across baseline body mass index subgroups. *Diabetes, Obes. Metab.*, **21** (6), 1340–1348.
74. Rohm, I., Kretzschmar, D., Pistulli, R., Franz, M., Schulze, P.C., Stumpf, C., and Yilmaz, A. (2016) Impact of Ivabradine on Inflammatory Markers in Chronic Heart Failure. *J. Immunol. Res.*, **2016**, 1–12.
75. Alsalame, H. "Study Effects of Nigella Sativa Seeds Oil in Some Physiological Parameters in Experimental Heart Failure Induced by Ivabradine in Male Rats." *Indian J. Forensic Med. Toxicol.*, **14** (3), 2609.
76. Nagaike, H., Ohara, M., Kohata, Y., Hiromura, M., Tomoyasu, M., Takada, M., Yamamoto, T., Hayashi, T., Fukui, T., and Hirano, T. (2019) Effect of Dulaglutide Versus Liraglutide on Glucose Variability, Oxidative Stress, and Endothelial Function in Type 2 Diabetes: A Prospective Study. *Diabetes Ther.*, **10** (1), 215–228.
77. Kastelein, J.J.P. (2014) Decade in review--dyslipidaemia: Resurgence of targets and compounds to treat dyslipidaemia. *Nat. Rev. Cardiol.*, **11** (11), 629–31.
78. Gerstein, H.C., Colhoun, H.M., Dagenais, G.R., Diaz, R., Lakshmanan, M., Pais, P., Probstfield, J., Botros, F.T., Riddle, M.C., Rydén, L., Xavier, D., Atisso, C.M., Dyal, L., Hall, S., Rao-Melacini, P., Wong, G., Avezum, A., Basile, J., Chung, N., Conget, I., Cushman, W.C., Franek, E., Hancu, N., Hanefeld, M., Holt, S., Jansky, P., Keltai, M., Lanas, F., Leiter, L.A., Lopez-Jaramillo, P., Cardona Munoz, E.G., Pirags, V., Pogosova, N., Raubenheimer, P.J., Shaw, J.E., Sheu, W.H.-H., Temelkova-Kurktschiev, T., and REWIND Investigators (2019) Dulaglutide and renal outcomes in type 2 diabetes: an exploratory analysis of the REWIND randomised, placebo-controlled trial. *Lancet (London, England)*, **394** (10193), 131–138.
79. Daniels, A., Linz, D., van Bilsen, M., Rütten, H., Sadowski, T., Ruf, S., Juretschke, H.-P., Neumann-Haefelin, C., Munts, C., van der Vusse, G.J., and van Nieuwenhoven, F.A. (2012) Long-term severe diabetes only leads to mild cardiac diastolic dysfunction in Zucker diabetic fatty rats. *Eur. J. Heart Fail.*, **14** (2), 193–201.
80. Frustaci, A., Kajstura, J., Chimenti, C., Jakoniuk, I., Leri, A., Maseri, A., Nadal-Ginard, B., and Anversa, P. (2000) Myocardial cell death in human diabetes. *Circ. Res.*, **87** (12), 1123–32.
81. Connelly, K.A., Gilbert, R.E., and Krum, H. (2008) Letter by Connelly et al regarding article, "Diastolic stiffness of the failing diabetic heart: importance of fibrosis, advanced glycation end products, and myocyte resting tension". *Circulation*, **117** (23), e483; author reply e484.
82. Paulus, W.J., and Tschöpe, C. (2013) A novel paradigm for heart failure with preserved ejection fraction: comorbidities drive myocardial dysfunction and remodeling through coronary microvascular endothelial inflammation. *J. Am. Coll. Cardiol.*, **62** (4), 263–71.
83. Thent, Z.C., Lin, T.S., Das, S., and Zakaria, Z. (2012) Histological changes in the heart and the proximal aorta in experimental diabetic rats fed with Piper sarmentsoum. *African J. Tradit. Complement. Altern. Med. AJTCAM*, **9** (3), 396–404.
84. HAYAT, S.A., PATEL, B., KHATTAR, R.S., and MALIK, R.A. (2004) Diabetic cardiomyopathy: mechanisms, diagnosis and treatment. *Clin. Sci.*, **107** (6), 539–557.
85. Ikonomidis, I., Pavlidis, G., Thymis, J., Birba, D., Kalogeris, A., Kousathana, F., Kountouri, A., Balampanis, K., Parissis, J., Andreadou, I., Katogiannis, K., Dimitriadis, G., Bamias, A., Iliodromitis, E., and Lambadiari, V. (2020) Effects of Glucagon-Like Peptide-1 Receptor Agonists, Sodium-Glucose Cotransporter-2 Inhibitors, and Their Combination on Endothelial Glycocalyx, Arterial Function, and Myocardial Work Index in Patients With Type 2 Diabetes Mellitus After 12-Month Treatme. *J. Am. Heart Assoc.*, **9** (9), e015716.
86. Khaleel, H.K. (2019) Investigating the Histological Changes in Heart, Lung, Liver and Kidney of Male Albino Mice Treated with Ivabradine. *Baghdad Sci. J.*, **16** (3(Suppl.)), 0719.