Glucose Transporter 4 and Peroxisome Proliferator-Activated Receptor-Alpha Overexpression Association With Cardioprotective Effects of Myoinositol and Metformin Combination in Type 2 Diabetic Rat Model

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Abstract

Background: Type 2 diabetes mellitus (DM) has many complications associated with increased morbidity and mortality. Cardiovascular complications are one of these serious complications. Therefore, there is a need for early and effective management to hamper them. This study aimed to evaluate the cardioprotective efficacy of metformin and myoinositol combination through exploring the associated underlying mechanisms in type 2 DM rat model.

Methods: Type 2 DM model was induced in rats by streptozotocin and high-fat diet. Rats were treated with metformin (150 mg/kg) and/ or myoinositol (50 mg/kg) by oral gavage once daily for 4 weeks. Immunohistochemistry was used to assess the drugs’ cardioprotective efficacy by estimating troponin T, nuclear factor kappa B (NF-κB) and tumor necrosis factor alpha (TNF-α). Quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay were utilized to investigate peroxisome proliferator-activated receptor-α (PPARα) and glucose transporter 4 (GLUT-4) expression levels in the skeletal muscles, respectively.

Results: This study found that metformin and myoinositol combination was associated with GLUT-4 and PPARα overexpression, together with better lipid profile than metformin alone in diabetic rats. Additionally, the combination of both drugs improved troponin C and decreased creatine kinase MB isoenzyme, NF-κB and TNF-α cardiac levels.

Conclusion: The current study indicated that myoinositol in combination with metformin had better cardioprotective effect than metformin alone in type 2 DM. This favorable effect was exhibited through upregulation of GLUT-4 and PPARα receptors expression in skeletal muscles, thus increasing insulin sensitivity and improving lipid profile.

Keywords: Type 2 diabetes mellitus; Metformin; Myoinositol; PPARα; GLUT-4; NF-κB; TNF-α

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. It is associated with microvascular and macrovascular complications, including retinopathy, nephropathy, and neuropathy, as well as cerebrovascular disease, ischemic heart disease, and peripheral artery disease [1]. Type 2 DM is the most prevalent type, representing 90-95% of DM and occurs due to a progressive loss of β-cell insulin secretion frequency on the background of insulin resistance [2].

DM is related with multiple cardiovascular complications including atherosclerosis, stroke, ischemic heart disease and peripheral vascular disease. It is also associated with cardiomyopathy which is one of the most frequent and common chronic complication that happens in type 2 DM [3]. It occurs in 12% of the diabetic patients leading to heart failure and death. Nearly one half of all deaths in diabetic patients are due to cardio-vascular diseases [4]. Therefore, one of the targets in diabetes treatment is controlling blood glucose level, thus preventing various cardiovascular insults. The management goals
for type 2 DM are to prevent or delay complications and maintain quality of life. This requires early diagnosis and treatment of hyperglycemia in addition to management of cardiovascular risk factors [5]. Although the main pathogenesis of type 2 DM is insulin resistance, there are only two of all antidiabetic drugs (thiazolidinedione and metformin) that act by increasing insulin sensitivity [2].

Glucose transporter 4 (GLUT-4) is a major mediator of glucose removal from the circulation and a key regulator of whole-body glucose homeostasis. It is highly expressed in adipose tissue and skeletal muscle. Decrease in GLUT-4 expression and/or decrease in its translocation in the skeletal muscle and adipose tissue lead to insulin resistance and propensity toward DM [6]. This peripheral insulin resistance causes pancreatic β cells to secrete more insulin, in a process known as compensatory hyperinsulinemia. However, together with insulin resistance, often there is β-cell depletion, which results in sustained hyperglycemia and type 2 DM [7].

Peroxisome proliferator-activated receptors (PPARs) are ligand activated transcription factors regulating important genes in cell differentiation and various metabolic processes, particularly lipid and glucose homeostasis [8]. The PPAR family consists of three isoforms: PPARα, PPARβ/δ and PPARγ that differ from each other in tissue distributions, ligand specificities and physiological roles [9].

PPARα is highly expressed in metabolically active tissues, such as liver, heart, skeletal muscle, intestinal mucosa, and brown adipose tissue. This receptor is involved in fatty acid metabolism and its activation lowers lipid levels [10]. It also has a role in glucose homeostasis and insulin resistance development [11]. In the liver, PPARα stimulates gluconeogenesis and ketone body synthesis and is involved in lipoprotein assembly. In the heart, it participates in the regulation of the metabolic switch between glucose and lipid oxidation [12]. This led to the development of many synthetic PPARs agonists for the treatment of different clinical outcomes over the past several decades [13]. For example, PPARα activators such as fenofibrate and clofibrate are useful drugs for the treatment of dyslipidemia through increasing high-density lipoprotein (HDL) and decreasing triglycerides (TGs) with no effects on low-density lipoprotein (LDL). PPARγ is a target of synthetic insulin sensitizers thiazolidinediones such as pioglitazone and rosiglitazone, which are used in the treatment of type 2 DM. Dual agonists of PPARα/γ, such as glitazar, have been developed and have recently become available for the combined treatment of type 2 DM and dyslipidemia [14].

The underlying pathophysiological features and subcellular mechanisms responsible for the development of diabetic cardiomyopathy are not completely understood. Metabolic abnormalities and overproduction of reactive oxygen and nitrogen species may trigger various intracellular pathways and alter myocardial expression of different genes. Increased nitro-oxidative stress, cardiomyocyte hypertrophy, profibrotic signaling, and myocardial remodeling along with apoptotic processes have been reported to play a critical role in the development of cardiomyopathy in both types of DM [15]. Persistent hyperglycemia in diabetes provokes excessive production of reactive oxygen species (ROS) and inflammation which play a key role in diabetic complications [16, 17]. Hyperglycemia induces glucose auto-oxidation and surplus generation of ROS. Hyperlipidemia can also increase ROS production through stimulating nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and inducing leakage of the mitochondrial electron transport chain [18]. Excess ROS activates protein kinase C and subsequently nuclear factor kappa B (NF-κB), leading to myocardial injury [16, 17]. NF-κB is a redox-sensitive protein complexes with a central role in inflammation. Activated NF-κB promotes the transcription and release of inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) and thereby provokes myocardial inflammation [19].

Metformin is one of the well-known drugs used in type 2 DM and has shown advantages in controlling hyperglycemia and decreasing microvascular complications. However, its role in opposing cardiovascular consequences and cardiovascular mortality needs more investigations [4]. Curiously, a clinical study in 2019 has suggested a significant protective role for metformin/curemic combination against type I DM-associated cardiac complications [20].

Inositol is a six carbon cyclitol existing under nine stereoisomeric forms depending on the spatial orientation of its six hydroxyl groups. It is the predominant form of inositol present in nature and in our food [21]. Myoinositol is a precursor in the phosphatidylinositol cycle and a source of several second messengers including diacylglycerol, which regulates some members of the protein kinase C family, myoinositol-1,4,5-triphosphate, which modifies intracellular calcium levels, and phosphatidylinositol-3,4,5-biphosphate, that is involved in the signal transduction. It is a component of cell membranes and is an essential nutrient required by the human cells for growth and survival [22].

The present study was conducted to investigate the cardioprotective effect of metformin and myoinositol either alone or in combination in high-fat diet (HFD), streptozotocin (STZ)-induced diabetic rats with the following assessments: fasting blood glucose (FBG), insulin level and lipid profile, along with measurement of PPARα and GLUT-4 expression levels in the skeletal muscles, cardiac levels of troponin C, nuclear factor kappa, TNF-α, and finally detect both the histopathological and immunohistochemical changes of the diabetic rats’ heart.

Materials and Methods

Animals’ protocols

This study was approved by Ain Shams University Research Committee, Faculty of Medicine. This study was performed in accordance with the ethical standards of Ain Shams University Research Committee (ethics committee reference number: 00017585).

Thirty adult male Wistar rats, purchased from an animal farm in Helwan, Cairo, Egypt, and initially weighing 150 - 200 g (age about 10 weeks) were used in the study. Animals were kept at the animal house of the Research Center at the Bilharz-ial Research Unit, Faculty of Medicine, Ain Shams University, under standard conditions of boarding: 12 h light/dark cycles, fed on standard chow, and water ad-libitum, throughout the
whole period of the study (7 weeks). Rats were left to acclimate for a week before any intervention. The animals were randomly divided into five groups, each containing six rats as the following: group 1: control rats received 0.1 M citrate buffer (pH 4.5); group 2: diabetic control rats received HFD and STZ (40 mg/kg) dissolved in 0.1 M citrate buffer (pH 4.5); group 3: myoinositol-treated diabetic rats received myoinositol (50 mg/kg) dissolved in water; group 4: metformin-treated diabetic rats received metformin (150 mg/kg) dissolved in water; group 5: myoinositol + metformin-treated diabetic rats received metformin (150 mg/kg) + myoinositol (50 mg/kg) dissolved in water. The test drugs were administered by gavage once daily for 4 weeks. The age of the animals at the beginning of drug administration was about 14 weeks. Myoinositol (Sigma Aldrich, Germany) was supplied as white powder and was dissolved in water. Metformin (Amoun, Egypt) was supplied as white powder and was dissolved in water. The doses were chosen according to the corresponding human average therapeutic doses used by US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER) [23].

**Sample collection**

Blood samples were collected, from the fasting animals, from the tail vein for biochemical analysis and skeletal muscle tissues (gracilis muscle) were excised rapidly and homogenate was prepared for GLUT-4 estimation and RNA isolation after 7 weeks from the beginning of the experiment (diet administration).

**Biochemical analysis**

The FBG was measured by glucose assay kit (Sigma-Aldrich, USA) at 7 weeks. Fasting serum insulin (INS) was measured by enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, USA) after 7 weeks according to the manufacturers’ instructions. Lipid profile measurements (TGs, total cholesterol, LDL and HDL serum levels) were done by triglycerides enzymatic assay kit (XpressBio, USA), cholesterol assay kit (BioChain, USA) and HDL assay kit (BioChain, USA), respectively. LDL was calculated using Friedewald equation after 7 weeks from the beginning of the experiment.

**Measurement of GLUT-4 concentration from skeletal muscle using ELISA**

Estimation of GLUT-4 was done using rat GLUT4 ELISA kit (Biospes, China) after 7 weeks from the beginning of the experiment.

**Total RNA extraction from skeletal muscle tissues**

Total RNA extraction was performed by miRNeasy mini kit (Qiagen, Germany) (cat no. 217004), then the RNA concentration and purity were measured by a NanoDrop 1000 spectrophotometer [24]. Reverse transcription of the extracted RNA was done using miScript II RT kit (cat no. 218161) (Qiagen, Germany) producing cDNA.

**Quantitative real-time polymerase chain reaction (qRT-PCR) for PPARα gene expression in skeletal muscle tissues**

The quantification of levels was amplified from mRNA using a QuantiTect primer assay primer assays (Rn_Ppara_1_SG QuantiTect primer assay, cat no: 249900, assay ID: QT00176575), and the ACTB primer sequence was used as a housekeeper gene. Quantitative PCR was carried out using QuantiTect SYBR Green PCR kit (Qiagen, Germany) (cat no. 204141) according to manufacturer’s instructions. All samples were analyzed using the five-plex Rotor-Gene PCR analyzer (Qiagen, Germany). Forty cycles were used, each with denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 70 °C for 30 s.

The expression of PPARα was defined based on the cycle threshold (Ct). The relative expression levels of PPARα were calculated as 2^-Ct after normalization to the expression of ACTB content in each examined tissue sample [25]. Melting curves were performed to check specificity of PCR products.

**Histopathological processing of the heart**

The heart was dissected longitudinally, and the whole left ventricle was fixed in 10% neutral buffered formalin, dehydrated in ascending grades of alcohol, cleared in xylol, embedded in paraffin blocks, and cut into 5 µm sections. The sections were dewaxed in xylol, rehydrated in descending grades of alcohol, rinsed with distilled water, and stained with hematoxylin and eosin (H&E) to evaluate the histological structure of the heart and Masson’s trichrome stain to visualize the distribution and content of the collagen fibers.

**Immunohistochemical study of the heart**

Immunohistochemistry staining was performed with specific primary antibodies, according to the manufacturer’s guidelines. Briefly, sections were subjected to target antigen retrieval with a citrate buffer, blocked with serum for 30 min, and then...
incubated with the anti-NF-κB p105 antibody produced in rabbit (1:50 - 1:100, SAB4501986; Sigma-Aldrich), anti-troponin T antibody, clone 9C2.1 (1:50 dilution, MABT368; Sigma-Aldrich), and anti-TNF-α antibody (1:50 - 1:200, SAB5700627; Sigma-Aldrich) for 1 h. Sections were washed with PBS, then incubated with biotinylated secondary antibodies for 30 min. Negative controls were prepared by omitting the primary antibody step. The site of antigen-antibody binding was visualized by 3,3′-diaminobenzidine (DAB) staining which appeared as dark-brown. Sections were counterstained with hematoxylin for nuclear staining.

**Morphometric analysis of heart histological sections**

The area percentage of collagen fibers in Masson’s trichrome-stained sections as well as the area percentage of the protein expression for immunohistochemical analysis (NF-κB, troponin T and TNF-α) was quantified using image analyzer Leica Q win V.3 program installed on a computer connected to a Leica DM2500 microscope (Wetzlar, Germany).

The image analyzer was first automatically calibrated to change pixels (measurement units produced by the image analyzer) into micrometer units in a way that one pixel was equal to 0.145 μm. This was done in five different non-overlapping stained sections using objective lens × 40 (five high-power fields, × 40/section) from different rats in each group. The reading of each rat was considered as a single variable. The data were then subjected to statistical analysis.

**Statistical analysis**

Analysis of variance (ANOVA) followed by the post-hoc and Kruskal-Wallis tests were conducted for the comparison between four groups through Social Science version 20 (SPSS Software, SPSS Inc., Chicago, IL, USA) before graphing. The results were expressed as mean, standard deviation, median and mean rank, and the level of statistical significance was set at P < 0.05.

**Results**

**Myoinositol and metformin combination improved glycemic control, lipid profile and CK-MB in diabetic rats**

Mean levels of serum FBG (mg/dL), insulin (µIU/mL), CK-MB (mg/dL), total cholesterol (mg/dL), TGs (mg/dL), LDL (mg/dL) and HDL (mg/dL) in the different animal groups were estimated. Diabetic group showed a highly significant increase in serum FBG, insulin, CK-MB, total cholesterol, TGs and LDL levels (305.8 ± 59.3, 18.6 ± 2, 54 ± 8.6, 118.6 ± 6.4, 111.5 ± 7.1, and 64 ± 2.3, respectively) compared to control group (103.6 ± 11.4, 2.6 ± 0.2, 10 ± 1.7, 41 ± 2.3, 37 ± 2.7, and 22 ± 3, respectively, P < 0.01), while HDL level was significantly lower in the diabetic group (24.8 ± 1.1) as compared to the control (36 ± 1.4) (P < 0.01). Myoinositol (164 ± 24.1, 9.4 ± 0.7, 40 ± 5.4, 62 ± 3.8, 54.8 ± 2.9, and 33.8 ± 1.3), metformin (184 ± 85.6, 6.8 ± 0.8, 24.8 ± 6.8, 50.8 ± 3.9, 47.5 ± 1.3, and 31.6 ± 1.9) and myoinositol + metformin-treated groups (174 ± 59.2, 3 ± 0.2, 19.4 ± 1.7, 39.1 ± 1.5, 34.6 ± 2.1, and 21.5 ± 1.9) showed significant reduction of FBG, insulin, CK-MB, total cholesterol, TGs and LDL levels compared to diabetic group (P < 0.01); however, myoinositol (32.3 ± 0.8), metformin (33.5 ± 0.5) and myoinositol + metformin-treated (36 ± 0.8) groups showed significant increase in HDL level compared to the diabetic group (P < 0.01). Myoinositol + metformin treatment significantly improved the fasting insulin level and lipid profile in diabetic rats better than treatment with either drug alone (P < 0.01) as represented in Table 1.

**GLUT-4 protein level increased in skeletal muscle cells after treatment with myoinositol and metformin combination**

In this experiment, ELISA was performed for detecting the concentration of GLUT-4 (µg/g) in skeletal muscle cells in the different animal groups. The mean levels of GLUT-4 in skeletal muscle cells in the diabetic group showed a significant decrease compared to the control (90.3 ± 10.1 and 137.7 ± 6.8, respectively, P < 0.05). However, treatment with myoinositol, metformin and both drugs combined together resulted in a significant increase of GLUT-4 levels (162.1 ± 32.4, 167.5 ± 37.8, and 190 ± 20.5, respectively) compared to the diabetic animals as shown in Figure 1A.

**PPARα overexpressed in skeletal muscle tissue of diabetic rats treated with myoinositol and metformin combination**

In this experiment, qRT-PCR was performed for detecting any possible changes in PPARα. The mRNA levels in skeletal muscle tissue homogenate were prepared from the different test groups. The relative quantification (RQ) of PPARα in skeletal muscle cells in diabetic group (median RQ 0.33) showed a significant decrease compared to the control (median RQ 21.38). On the other hand, myoinositol treatment (median RQ 5.74), metformin treatment (median RQ 17.19) and treatment with both myoinositol and metformin (median RQ 16.22) resulted in a significant increase of PPARα expression compared to the diabetic group (median RQ 0.33). Combined treatment significantly increased PPARα expression more than myoinositol treatment only (P < 0.01, Fig. 1B).

**Negative correlation of GLUT-4 protein and PPARα mRNA with different biochemical parameters**

There was non-significant direct correlation between GLUT-4 and PPARα expression levels (r = 0.34 with P = 0.063). GLUT-4 protein (µg/g) and PPARα mRNA expression (RQ) had a significant inverse correlation with plasma insulin level (µIU/mL), total blood cholesterol (mg/dL), TGs (mg/dL), and LDL (mg/dL), while there was a significant direct
correlation with HDL levels (mg/dL) \( (P < 0.01) \) as shown in Table 2.

Heart histopathological picture improved after combined intake of both myoinositol and metformin in diabetic rats

Examination of H&E-stained sections revealed that the myocardium of the diabetic rats showed deterioration of its architecture as compared to the control group; there were loss of striations, fatty infiltration together with areas of focal degeneration. Furthermore, the cardiac muscle fibers were widely separated from each other by inflammatory cell infiltrations, edema, hemorrhage and dilated congested blood vessels (Fig. 2b, c). Myoinositol-treated group showed some improvement in the myocardium architecture as compared to the diabetic group; however, some myocardial cells still appeared deeply acidophilic, and others showed focal necrosis (Fig. 2d). Metformin-treated group also showed some improvement apart from intramyocardial fat infiltration (Fig. 2e). However, treatment of diabetic rats with both metformin and myoinositol resulted in marked improvement of the myocardial structure as compared to the control normal rats with no evidence of degeneration, necrosis, or inflammation (Fig. 2f).

Myoinositol and metformin combination decreased myocardial fibrosis in HFD fed STZ-induced diabetic rats

As presented in Figures 3 and 4A, the Masson’s trichrome-stained sections revealed apparent fibrosis (%) within the myocardium in the diabetic group. The collagen area percentage was measured as 2.77 ± 0.83, which was significantly increased as compared to the control group \( (P < 0.05) \) while myoinositol treatment and metformin treatment showed few collagen fibers in between cardiac muscle fibers which resulted in a significant decrease in the collagen area percentage \( (1.62 ± 0.89 \text{ and } 1.05 ± 0.83, \text{ respectively}) \) as compared to diabetic rats. However, the collagen area percentage was significantly decreased when diabetic rats were treated with both myoinositol and metformin as compared to treatment with either myoinositol or metformin alone \( (0.53 ± 0.16, \text{ which is } P < 0.01) \).

Myoinositol and metformin combination improved cardiac troponin T with down-regulation of NF-κB p105 and TNF-α in the heart of the diabetic rats

Immunostaining for troponin T (%) showed marked positive reaction in normal myocardium as shown in Figures 4B and 5. However, it showed decreased staining in necrotic myocardium in the diabetic rats \( (25.31 ± 2.89) \) as compared to the control group \( (61.9 ± 4.9) \) \( (P < 0.05) \). Treatment of both antidiabetic drugs markedly improved the positive immunostaining of cardiac troponin T \( (53.96 ± 4.29) \) as compared to treatment of each drug alone \( (35 ± 3.4 \text{ and } 32.2 ± 4.8, \text{ respectively}) \) \( (P < 0.01) \).

Immunohistochemical staining for the inflammatory markers (NF-κB p105 and TNF-α) in the heart was performed in all
groups as shown in Figures 5 and 6. Low expression of NF-κB p105 and TNF-α (%) was observed in the heart of the control group, and this was quantified as area percentage of protein expression (median 0.5 and 0.3, respectively). Diabetic rats showed marked positive cytoplasmic reaction for NF-κB and TNF-α that represented a highly significant increase as compared to the control group (median 7.2 and 1.3, respectively) (P < 0.05). Treatment with both myoinositol and metformin resulted in a high significant decrease in NF-κB reaction and TNF-α expression (median 0.46 and 0.04, respectively) than treatment with metformin alone (median 0.64 and 0.15, respectively) (P < 0.01).

Discussion

Development of insulin resistance characterizes the key systemic abnormality in the pathogenesis of type 2 DM [26]. Cardiovascular diseases are one of the most common diabetes-related complications. They generally affect nearly 32.2% of type 2 DM patients and represent the main cause of death in them [27].

In the current study, we established HFD, STZ-induced diabetic rat model to assess the effect of metformin and/or myoinositol on PPARα and GLUT-4 expression levels and their correlation with FBG, insulin level and lipid profile.

The results of the present work exhibited that administration of metformin in combination with myoinositol improved insulin resistance as shown by the significant reduction of FBG, insulin, CK-MB, total cholesterol, TGs and LDL levels, and the significant increase in HDL level versus diabetic group as well as the significant rise of GLUT-4 levels and PPARα expression.

Table 2. Correlations Between GLUT-4 Level, PPARα RQs, FBG, Insulin Levels and Lipid Profile Among All the Studied Groups

|                        | FBG (mg/dL) | Plasma insulin level (µIU/mL) | Total cholesterol (mg/dL) | Triglyceride (mg/dL) | HDL (mg/dL) | LDL (mg/dL) |
|------------------------|-------------|-----------------------------|--------------------------|---------------------|-------------|-------------|
| GLUT-4 (µg/g)          |             |                             |                          |                     |             |             |
| Correlation coefficient| -0.213      | -0.457*                     | -0.590**                 | -0.595**            | 0.527**     | -0.500**    |
| Sig. (two-tailed)      | 0.260       | 0.011                       | 0.001                    | 0.001               | 0.003       | 0.005       |
| PPARα RQs              |             |                             |                          |                     |             |             |
| Correlation coefficient| -0.663**    | -0.856**                    | -0.829**                 | -0.789**            | 0.819**     | -0.803**    |
| Sig. (two-tailed)      | 0.000       | 0.000                       | 0.000                    | 0.000               | 0.000       | 0.000       |

Data are presented as r-value. Person’s correlation test was used. *Correlation is significant at the 0.05 level (two-tailed). **Correlation is significant at the 0.01 level (two-tailed). GLUT-4: glucose transporter 4; PPARα: peroxisome proliferator-activated receptor-alpha; RQs: relative quantifications; FBG: fasting blood glucose; HDL: high-density lipoprotein; LDL: low-density lipoprotein.
For many decades, insulin resistance and type 2 DM have been studied and as well known, insulin resistance is a predictor of potential development of type 2 DM [28].

Insulin resistance and type 2 DM are associated with many dyslipidemia features including reduced HDL cholesterol, increased levels of LDL, TGs, total cholesterol and very low-density lipoprotein that are associated with an increased risk of atherosclerotic cardiovascular disease [29, 30].

In the present work, there were significant hypertriglyceridemia, hypercholesterolemia, elevation of LDL cholesterol and reduction of HDL cholesterol in the diabetic group compared to the control group. The antihyperlipidemic of myoinositol and metformin in this study was observed by the significant reduction of both total and LDL cholesterol as well as
TGs and elevation of the HDL level. Our results showed that combined treatment with both drugs had a more lipid lowering effect than each alone, and this effect was additive and better regarding the lipid profile control.

These results were in accordance with the studies by Muscogiuri et al and Iuorno et al, who found that systemic myoinositol supplementation improved systolic blood pressure, insulin resistance, cholesterol, and TGs levels in postmenopausal women with metabolic syndrome [31, 32]. Our results were also concomitant with the study by Karamchand et al, whose results clearly indicated the beneficial effect of metformin on lipid profile of type 2 DM, hence concluding that metformin had the ability to correct dyslipidemia in type 2 diabetic patients [30].

Expression of GLUT-4 was noticed in the tissues possessing insulin stimulated glucose transport as adipose tissues and skeletal and cardiac muscle as detected by Szablewski et al [33]. Knockout study of skeletal muscle and adipose tissue specific GLUT-4 mice showed severe insulin resistance and glucose intolerance at an early age [34].

Our results showed that the level of GLUT-4 was significantly lowered in the diabetic rats compared to the control, yet

Figure 4. Effect of tested drugs on myocardial fibrosis and troponin T percentage area in cardiac muscle cells in HFD-fed STZ-induced diabetic rats. (A) Diabetic group has a significantly the highest collagen percentage area. (B) Diabetic group has a significantly the lowest troponin positive cells while highest percentage in both myoinositol and metformin treated group is observed.

aP < 0.01, compared with normal control values. bP < 0.01, compared with diabetic control values. cP < 0.01, compared with myoinositol, dP < 0.01, compared metformin ttt. HFD: high-fat diet; STZ: streptozotocin.

Figure 5. Immunohistochemical stains of the cardiac muscle. Representative immunoperoxidase images showing expression of troponin T, TNF-α and NF-κB p105 in the myocardium of normal, diabetic and the three treated groups. Arrows indicate positive brown sites where the immune marker is expressed. Arrowheads indicate areas of negative immune reaction (avidin-biotin peroxidase, × 400). TNF-α: tumor necrosis factor alpha; NF-κB: nuclear factor kappa B.
its level increased with treatment with either myoinositol or metformin and was more pronounced with combined myoinositol and metformin treatment, resulting from the improvement of insulin sensitivity by the combined treatment. Metformin could reduce hyperglycemia also by improving peripheral insulin sensitivity and increasing glucose uptake into skeletal muscle cells [35]. On the other hand, Lee et al reported that metformin chronically induced Cbl-associated protein expression via AMP-activated protein kinase, and modulated GLUT-4 translocation [36].

The current results regarding myoinositol agreed with the study by Bevilacqua and Bizzarri, who found that both types of inositol phosphoglycans (containing myoinositol and D-chiro inositol) acted as second messengers downstream of insulin receptors, exerting an insulin mimetic activity. When administered to normal or diabetic rats, they reduced hyperglycemia in a dose-dependent fashion and encouraged muscular glycogenesis [37].

Since both metformin and myoinositol could increase GLUT-4 by different mechanisms, their effect could be additive as observed in our work.

The current study found that PPARα was down expressed in the diabetic rats as compared to both the control and treated groups. Interestingly, PPARα expression was significantly upregulated after treatment with metformin and myoinositol combined together as compared to either metformin or myoinositol alone. Moreover, the overexpression of PPARα was associated with decrease in TGs, total cholesterol and LDL levels and increase in HDL levels. Therefore, this study postulated that combined treatment with both metformin and myoinositol increased insulin sensitivity and improved lipid profile through upregulation of GLUT-4 and PPARα receptors expression in skeletal muscles. Many evidences had indicated that hypertriglyceridermia caused an excess of free fatty acids, which could increase insulin resistance “lipotoxicity” that is a vital pathogenetic issue clearly related with peripheral tissue insulin resistance and islet β-cell dysfunction [38]. Feng et al reported that PPARα agonists may protect against the cardiovascular complications of prediabetes and DM through decreasing hypertriglyceridermia, improving insulin sensitivity and β-cell function [39].

Furthermore, this study elucidates the protective role of this drug combination against the development of cardiac injury in diabetic rats. We evaluated the cardiac levels of troponin C, nuclear factor kappa, TNF-α, and the histopathological changes that occurred in the cardiac muscles. We revealed that the combined treatment with both myoinositol and metformin triggered marked improvement of the cardiac histopathological changes as detected by the significant improvement of the myocardial structure and the significant decrease of degeneration, necrosis, or inflammation and the collagen area percentage against diabetic group. A marked improvement of the positive immunostaining of cardiac troponin T with significant decrease in NF-κB and TNF-α expression was observed.

Histopathological changes of the myocardium of the diabetic rats showed areas of necrosis, fatty infiltrations, mononuclear cellular infiltrations, and congestion. The improvement of the cardiac architecture in the present study was better in combined metformin and myoinositol treatment than in metformin or myoinositol-treated groups. These results were in accordance with Roslan et al, who suggested the occurrence of cardiomyopathy induced by diabetes [40]. Hyperglycemia or
unstable glycemic metabolism leads to activation of leukocyte and recruitment of inflammatory neutrophils, monocytes, and macrophages to the myocardium [41]. Additionally, the accumulation of collagen fibers in the heart interstitium is another common finding of diabetic cardiomyopathy resulting in the inability of the heart to contract and relax efficiently [42]. In the current work, myocardial fibrosis was noticed in the diabetic group, consistent with other studies in different animal models of diabetes [43]. The deposition of collagen fibers was significantly decreased in combined treatment of metformin and myoinositol suggesting their coordinated role in preventing diabetes-induced interstitial fibrosis. Levick and Widiapradja postulated that hyperglycemia induced activation of many signaling pathways including renin angiotensin system, kinases, cytokines, and non-coding RNA. These signaling pathways resulted in activation of TGF-β1 causing excessive secretion of extracellular matrix especially collagen type I by cardiac fibroblasts [44].

Our results displayed areas with loss of immune expression of cardiac troponin T (cTnT) in the heart of the diabetic rats indicating focal myocardial necrosis. Treatment with combined metformin and myoinositol demonstrated marked significant increase in immune reaction for cTnT. Fishbein et al designated that immunohistochemical staining for cTnI and cTnT might be more sensitive than routine H&E staining for the diagnosis of myocardial necrosis in experimental animals as well as autopsy of human hearts [45].

In addition, many studies indicated that cardiomyopathy associated with DM results from myocardial inflammation. These proinflammatory responses and chronic low-grade inflammation that happen within cardiomyocytes are reactions to hyperglycemia and dyslipidemia that take place in insulin resistance and DM [46] as hyperglycemia triggers the production of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and TNF-α, through activating the NF-κB signaling pathway [47]. In the present study, it was observed that immunostaining for TNF-α was significantly increased in the myocardium of the diabetic rats and thereby activating the NF-κB signaling cascade which showed also increased immune reactions as compared to the control group. Administration of both antidiabetic drugs in combination could inhibit this increase in TNF-α and NF-κB immune reactivity in the cardiac tissue, thus ameliorating cardiac inflammation. Similarly, Saklani et al proposed that TNF-α is essential for pathogenesis of diabetic cardiomyopathy [48].

Conclusion

Adding myoinositol to metformin was associated with GLUT-4 and PPARα overexpression in skeletal muscle tissue. This may contribute to increasing insulin sensitivity with better glycemcic control and improving dyslipidemia. The combined therapy myoinositol plus metformin could be a promising new strategy to improve the metabolic profile in obese diabetic patients, thus reducing the risk for the various cardiovascular events.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Informed Consent

Not applicable.

Author Contributions

All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. They have made substantial contributions and roles in this manuscript. Dalia Abdel-Wahab Mohamed, Dina Sayed Abdulrahim, Yomna M. Tamim and Asmaa A. Abozeid have a contribution in conception and design, acquisition of data, analysis and interpretation of data and drafting the manuscript. Wael M. Elayat, Mohamed F. Abdel-Salam, and Nahla Mohamed Teama contributed to analysis, interpretation of data and revising.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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