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Anti-diabetic effect of cotreatment with resveratrol and pioglitazone in diabetic rats

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Abstract. – OBJECTIVE: Limitations and side effects associated with current anti-diabetic treatments have necessitated the search for new therapeutic alternatives. This study aimed to explore the combined use of resveratrol (RVT) and established anti-diabetic drug pioglitazone (PGZ) against streptozotocin (STZ)-induced diabetes mellitus (DM).

MATERIALS AND METHODS: STZ was supplemented daily to Sprague-Dawley rats to induce DM. The synergistic effect of the RVT (20 mg/kg) and PGZ (0.65 mg/kg) on DM complications was evaluated after 8 weeks of treatment. Biochemical analyses were performed to evaluate the effectiveness of our treatment on glucose level, insulin sensitivity, lipid disturbances, oxidative mediators and inflammatory markers.

RESULTS: STZ induced DM onset that is accompanied with elevated diabetic markers, lipid disturbances, remarkable oxidative damage and hyper-inflammation. The PGZ+RVT combination has the best effect as illustrated by significant ($p < 0.05$) decreases in fasting blood glucose, insulin, HbA1c and HOMA-IR levels. This combination attenuated ($p < 0.05$) lipid disturbances and their associated elevated atherogenic biomarkers. At the same time, treatments with PGZ+RVT exhibited an anti-inflammatory effect as it attenuated the increase in inflammatory parameters (CRP, TNF-α, IL-6). Also, it restored total antioxidant capacity and peroxisome proliferator-activated receptor (PPARγ) levels that decreased by STZ-DM induction.

CONCLUSIONS: This study provides PGZ+RVT as promising DM therapeutic alternative. This synergistic combination alleviates most of DM-related complications and insulin resistance.

Key Words:
Resveratrol, Pioglitazone, Anti-diabetic, Antioxidant, Anti-inflammatory.

Introduction

With a number of different etiologies, diabetes mellitus (DM) is a chronic metabolic syndrome that severely affects patients' life and elevates the risk of developing other chronic diseases[1-3]. Due to hypoinsulinism or insulin resistance (IR), DM is characterized by abnormal proteins, lipids, and carbohydrates metabolism[4]. International Diabetes Federation recent statistics reported that about 463 million adults (ages 20-79 years) have DM, many of which live in poverty and in developing countries. By 2045, this is expected to increase to 700 million cases[5].

Current synthetic antidiabetic drugs have many benefits. However, they are associated with many adverse side effects including hypoglycaemia, renal failure, diarrhea, vomiting, etc[6]. So, alternative antidiabetic therapies with minimal or no hazardous side effects are required[1]. Currently, compared to oral chemical hypoglycemic drugs, new bioactive compounds have been extracted

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from plants and have antidiabetic activity with more efficiency[4-7]. These bioactive compounds have multiple activities in insulin action, insulin production, or both[1,8].

Resveratrol (RVT) is phytochemical from polyphenolic compounds stilbene class. It isolated from red grape and can demonstrate strong antidiabetic activity[9]. Also in DM rats, it can protect against diabetic nephropathy while its administration has protective activities in oxidative stress and renal dysfunction[10]. By redox plasma membrane system activation, RVT can effectively restore cellular homeostasis11. In DM rats, RVT administration has resulted in decreased HbA1c concentration[12]. In DM animals, RVT antihyperglycemic efficacy was reported to be due to the stimulatory activity on intracellular glucose transportation and glucose uptake[9].

Peroxisome proliferator-activated receptors (PPARs) are important factors in stimulating many biologic processes, including cell differentiation and growth, immune response, insulin sensitivity, adipogenesis and lipid metabolism[13]. One ligand for PPAR-γ, one of thiazolidinediones (TZD), is pioglitazone (PGZ). It is effective oral anti-hyperglycaemic treatment with additional lipid and cardiovascular (CV) benefits that allows for T2DM patients successful management[14].

This study aimed to evaluate the anti-diabetic effects of RVT alone and in combination with PGZ including attenuation of glucose related parameters (blood glucose, glycosylated hemoglobin (HbA1c), insulin, homeostatic model assessment of insulin resistance (HOMA-IR)), lipid profile, anti-inflammatory and antioxidant activities.

Materials and Methods

Ethical Considerations

All experiments and animals involved in this study were carried out according to the National Research Council's suggested criteria for animal care and use15. The study protocol was approved by the Animal Ethics Committee of School of Graduate Studies, Jordan University.

Reagents and Materials

PGZ raw material (HCl powder, 98.5%; Lot # BWP200023) was obtained from Dar Al Dawa, Jordan. Deionized water (B#1207702) was purchased from Thermo Fisher Scientific, (Waltham, MA, USA). RVT (Enzo Life Sciences Inc., Farmingdale, NY, USA) and STZ [>95%; (bioXTra, London, UK), Lot # 18883-66-4] were purchased. All enzyme immune assay kits [including interleukin (IL)-6 and tumor necrosis factor-α (TNF-α)] were purchased from MyBioSource, (San Diego, CA, USA). The following instruments were used: Multiskan Go Spectrophotometer, Model 1510, Thermo Fisher, UKBath, Sonicator Crest model-175T (UltraSonics CORP), Sartorius analytical balance, and Centrifuge (Eppendorf 5417C).

Animals, Diabetic Induction, and Study Design

Sprague-Dawley male rats (220-300 g; n=45) were included from Animal house, School of Graduate Studies, Jordan University, Jordan. Under nutritional necessities and standardized environmental, all animals were housed with free access to food and water. Rats classified into five main groups (9/each group). G0 (Negative control) received just regular baseline animal. All other groups were subsequently intraperitoneally given a low amount of STZ (40 mg/kg bwt of freshly parepared STZ solution [32.25 mg/ml in 0.05 M sodium citrate buffer, pH 4.5)]. T2DM was obtained in animals when fasting blood glucose (FBG) was ≥120 mg/dL and non-FBG was ≥250 mg/dL. G1 was baseline DM animals, [G2 (PGZ (0.65mg/kg) alone), G3 RVT (20 mg/kg) alone) and G4 (PGZ (0.65mg/kg) and RVT (20 mg/kg)].

Evaluation of Diabetic Related, Lipid Profile, Anti-Inflammatory and Antioxidant Parameters

Before, during and after the treatment stage (about 6 weeks), cardiac puncture and blood were collected into plain tubes. Using fresh blood, FBG (SPINREACT, Girona, Spain) and HbA1c (Automatic protein analyzer, GenruiPA120) were analyzed. To obtain serum, remaining blood is left to clot and then centrifuged. Serum samples were preserved frozen for biochemical testing. Selenium, leptin, PPAR-γ, insulin, TNF-α IL-6, C-reactive protein (CRP) and total antioxidant capacity (TAC) (all from MyBioSource, Rat, ELISA Kits) and lipid profile including cholesterol, triglycerides, LDL-C, and HDL-C (all from SPINREACT) were analyzed using specialized kits and following the manufactures' detailed instructions. HOMA-IR was calculated from the following equation [fasting insulin (μ/ml) × fasting glucose (mmol/l)/22.5][16].

Statistical Analysis

Results are expressed as mean±standard error of the mean. $p < 0.05$ was considered to be statisti-

cally significant. For statistical analysis of the data obtained from various groups, one-way analysis of variance (ANOVA) was performed followed by Tukey-Kramer as post hoc test. Pearson's correlation test was used to assess the interrelationships between biomarkers. Statistical analysis software (SAS version 9, USA) was used to analyse the data.

Results

Weight Change, Food and Water Intake Between Untreated and Treated Rats

Owing to pancreas damage, STZ administration leads to DM onset. As a function of diets during the study, rats' body weight varied significantly ($p < 0.05$). Also compared to non-diabetic rats, feed and water intake elevated significantly ($p < 0.05$) in DM groups. With DM induction, liver weights significantly increased (G1) and this effect was attenuated in treatments groups (Table I).

Effect of Pioglitazone and Resveratrol on Diabetic Markers

Compared to normal non-diabetic rats (G0), STZ-induced DM manifested profound hyperglycemia and was associated with significantly ($p < 0.05$) increased FBG, serum insulin, HbA1c and HOMA-IR levels. Although PGZ and RVT treatments each alone decreased these diabetic parameters, the best effect was achieved by their combinations as PGZ+RVT treated group showed significant decrease in FBG, serum insulin, HbA1c and HOMA-IR levels compared to diabetic control group (G1) (Figure 1).

Effect on Lipid Profile and Atherogenic Biomarkers

In all experimental rats, Table II shows serum levels of total cholesterol, triglyceride, LDL-C and HDL-C. Compared with normal non-diabetic group, diabetic control group shown significant increase in cholesterol, triglyceride and LDL-C and significant decrease in HDL-C ($p < 0.05$). Consequently, rats with DM were also associated with significantly ($p < 0.05$) increased lipid-related atherogenic biomarkers including atherogenic index of plasma (AIP), atherogenic coefficient (AC) and cardiac risk ratio (CRR) (Table II). Treatment with both PGZ and RVT attenuated these lipid alterations and increased atherogenic biomarkers, the beast effective effect was reported in PGZ+RVT in combination treated group ($p < 0.05$ for all parameters compared to diabetic untreated rats) (Table II).

Effect on Inflammatory and Antioxidant Statuses

Compared to non-diabetic rats, DM inducing elevated ($p < 0.05$) levels of anti-inflammatory parameters including CRP, TNF-α and IL-6. Also, diabetic rats were associated with increased ($p < 0.05$) leptin serum levels. In contrast, DM induction causes significantly ($p < 0.05$) reduction in TAC and PPARϒ expression (Table III). After treatments with PGZ, RVT and PGZ+RVT these issues were ameliorated (significant difference varied according to the treatment type; Table III). As mentioned in previous DM related complications, the best effect on anti-inflammatory and antioxidant statuses was reported in PGZ+RVT

Table I. Body weight, food and water intakes, and liver weight of the study groups.

Variables	Non-Diabetic Control (n=9)	Streptozotocin-Induced Diabetic Groups			
		Control (n=9)	PGZ (n=9)	RVT (n=9)	PGZ+RVT (n=9)
Initial weight (g)	249±14	231.6±1.5	244.0±7.8	257.8±16.5	256.6±10.3
Final weight (g)	253.1±14.2	221.0±2.2	227.3±5.8	247.1±17.0	240.6±8.7
Δ Weight (g/day)	0.09±0.01	-0.25±0.03	-0.40±0.07[a]	-0.25±0.02	-0.38±0.19
Food intake (g/day)	0.32±0.01	0.50±0.0	0.96±0.02[a,b]	0.81±0.01[a,b]	0.90±0.01[a,b]
FER	27.58±3.14	-0.5±6.99[a]	-41.34±7.8[a]	-31.14±2.60	-43.10±21.6
Water intake (ml/day)	0.93±0.01	1.85±0.0[a]	2.89±0.04[a,b]	3.14±0.04[a,b]	3.14±0.04[a,b]
Liver weight (g)	7.52±0.24	8.77±0.27[a]	8.01±0.13	8.39±0.26	8.34±0.25
Liver index	3.05±0.20	3.97±0.13[a]	3.54±0.10	3.53±0.27	3.49±0.13

Values are the means±SEM. PGZ: pioglitazone; RVT: resveratrol; FER: food efficiency ratio (bodyweight change/100 g food intake); liver index: liver weight (g)/100 g final body weight. Values are significantly different at $p < 0.05$. a significantly different from non-diabetic control, b significantly different from diabetic control.

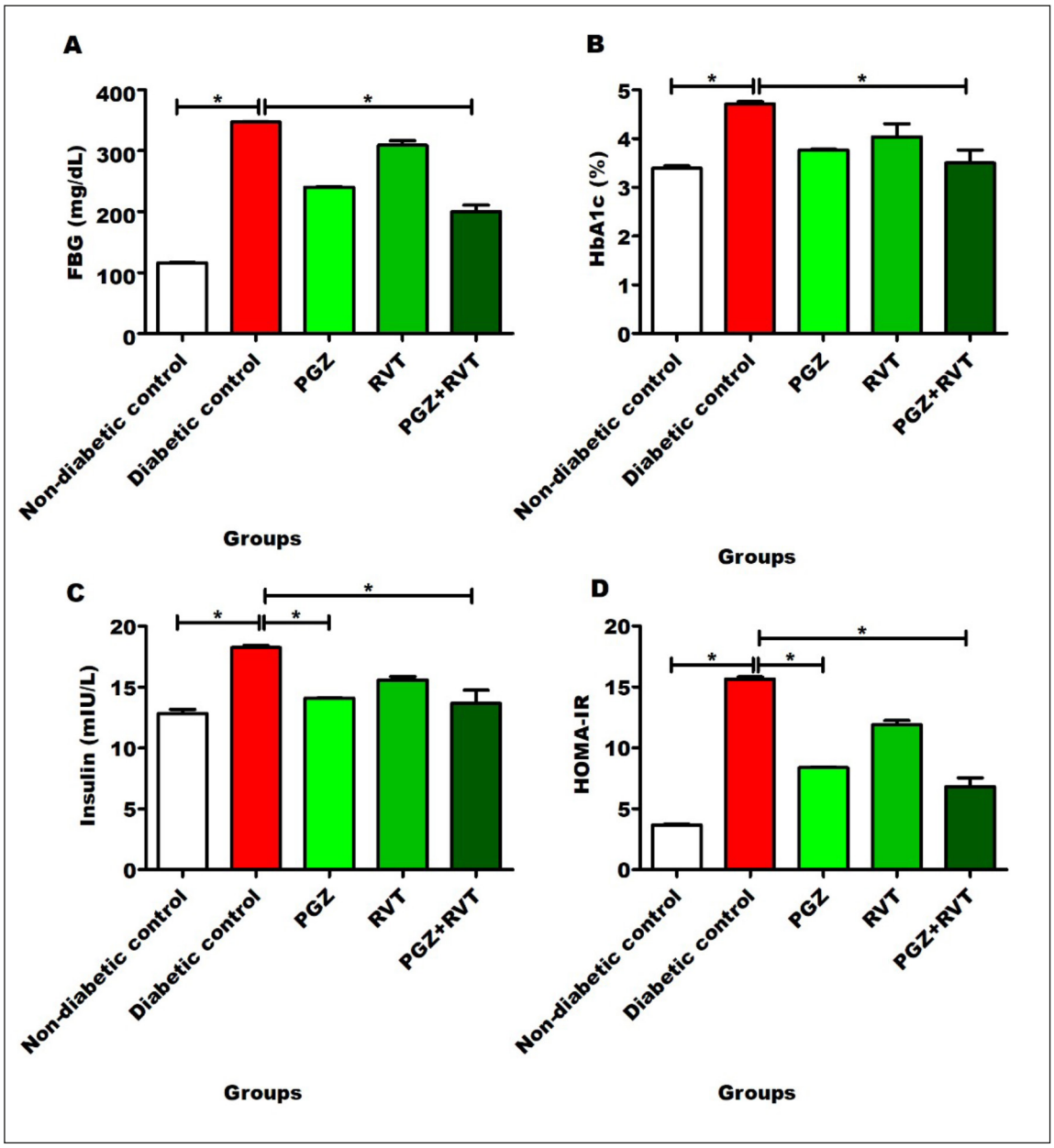

Figure 1. Diabetic markers between untreated and treated rats. PGZ+RVT combined therapy significantly attenuated increased (**A**) FBG, (**B**) HbA1c, (**C**) insulin, and (**D**) HOMA-IR levels. Values are the means±SEM. PGZ: pioglitazone; RVT: resveratrol; FBG: fasting blood glucose; HbA1C: glycosylated hemoglobin; HOMA-IR: homeostatic model assessment of insulin resistance. *means that values are significantly different at $p < 0.05$.

combination ($p < 0.05$ for all parameters compared to diabetic untreated rats) (Table III).

Discussion

Globally, DM has become public health issue that has reached epidemic proportions[17]. Reduced hyperglycemia may decrease the risk of developing DM-related chronic diseases[17]. Traditional DM pharmacological treatments have a wide range of toxic and adverse effects[18]. On the other hand, medicinal plants can have similar degree of efficacy without any side effects[17].

Even though RVT has a double effect on the processes that control the cell cycle and play an important role in determining cell viability[19], So, when treating LNCaP cells with resveratrol for 24

Table II. Lipid and atherogenic biomarkers between untreated and treated rats.

Variables	Non-Diabetic Control (n=9)	Streptozotocin-Induced Diabetic Groups			
		Control (n=9)	PGZ (n=9)	RVT (n=9)	PGZ+RVT (n=9)
Cholesterol (mg/dl)	54.9±0.3	106.2±1.0[a]	70.8±0.9	78.7±6.5	63.8±4.5[b]
LDL-C (mg/dl)	20.88±0.49	67.12±0.8[a]	36.55±1.05[a]	40.7±3.85[a]	25.26±1.97[b]
HDL-C (mg/dl)	27.62±0.13	12.13±0.3[a]	24.44±0.41[b]	21.7±1.03[a]	26.22±2.32[b]
Triglycerides (mg/dl)	39.5±1.1	134.8±1.1[a]	86.8±0.6[a]	100.4±16[a]	66.5±5.9[b]
LDL-C/HDL-C	0.76±0.02	5.56±0.15[a]	1.50±0.05[b]	1.86±0.11[a]	1.03±0.13[b]
Cholesterol/Triglycerides1.	40±0.04	0.79±0.01[a]	0.82±0.01[a]	0.91±0.13[a]	1.01±0.1
AIP	0.15±0.01	1.05±0.01[a]	0.55±0.01[a,b]	0.63±0.06[a]	0.40±0.05[b]
CRR	1.99±0.02	8.79±0.19[a]	2.90±0.06[b]	3.73±0.38[a]	2.49±0.13[b]
AC	0.99±0.02	7.79±0.19[a]	1.90±0.06[b]	2.73±0.38[a]	1.49±0.13[b]

Values are the means±SEM. PGZ: pioglitazone; RVT: resveratrol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; AIP: atherogenic index of plasma [log (Triglyceride/HDL-C)]; AC: atherogenic coefficient [TC-HDL-C/HDL-C]; CRR: cardiac risk ratio (TC/HDL-C). Values are significantly different at $p < 0.05$. a significantly different from non-diabetic control, b significantly different from diabetic control.

h, the dual effect of RVT on DNA synthesis was observed. At 5 to 10 μM it caused a 2-to 3-fold increase in DNA synthesis, and at >15 μM, it inhibited DNA synthesis[20]. The LNCaP cells, treated with RVT, are induced to enter into S phase, but subsequent progression through S phase is limited by the inhibitory effect of resveratrol on DNA synthesis, particularly at concentrations above 15 μM. Therefore, this unique ability of RVT to exert opposing effects on two important processes in cell cycle progression, induction of S phase and inhibition of DNA synthesis, may be responsible for its apoptotic and antiproliferative effects[20]. However, RVT has several pharmacological effects, including neuroprotective, anti-inflammatory, cardioprotective, antitumor, and antioxidant functions[21]. While lowering blood glucose, some studies[21] suggest that RVT improves both hepatic steatosis and insulin resistance. Recently, it has been suggested that RVT has synergistic effect with other established anti-diabetic drugs on type 2 DM[22]. Moreover, PGZ is anti-diabetic drug, PPARγ agonist that was discovered to uncouple unwanted ensuing hypoglycaemia, characteristic of other DM medications, from the lowering of blood glucose. While the α-glucosidase inhibitors effectively decreased systemic glucose and sulphonylureas and insulin regulate signalling from the pancreas to peripheral tissues, PGZ paved, in peripheral tissues, alternate path by increasing insulin responsiveness[23]. So, this study aimed to clarify hypoglycemic synergistic effects of RVT and/or PGZ as mono- and combined-therapy in STZ-induced DM in rats.

Table III. Anti-inflammatory and anti-oxidant parameters between untreated and treated rats.

Variables	Non-Diabetic Control (n=9)	Streptozotocin-Induced Diabetic Groups			
		Control (n=9)	PGZ (n=9)	RVT (n=9)	PGZ+RVT (n=9)
CRP (ng/ml)	2952±856	12188±316[a]	5146.1±316[a,b]	5221±1442[a,b]	3503±690
TNFα (pg/ml)	241.8±1.5	542.1±3.5[a]	402.0±3.3[a]	439.2±37.2[a]	356.8±4.0[b]
IL-6 (pg/ml)	20.89±0.03	92.71±1.33[a]	53.64±1.17	76.83±1.93[a]	25.8±1.99[b]
TAC (U/ml)	0.23±0.01	0.15±0.01[a]	0.18±0.01	0.17±0.02	0.22±0.01[b]
PPARϒ (pg/ml)	305.9±4.6	125.4±1.1[a]	231.8±0.9[b]	190.2±60.9	282.5±33.4
Leptin (ng/ml)	8.37±0.12	18.04±0.18[a]	9.43±0.02[b]	10.37±0.99[b]	8.77±0.62[b]
Selenium (μmol/ml)	0.34±0.12	0.31±0.14	0.10±0.06	0.17±0.08	0.23±0.08

Values are the means±SEM. PGZ: pioglitazone; RVT: resveratrol; CRP: C-reactive protein; TNFα: tumor necrosis α; IL-6: interleukin 6; TAC: total antioxidant capacity; PPARϒ: Peroxisome proliferator-activated receptor-gamma. Values are significantly different at $p < 0.05$. [a]significantly different from non-diabetic control, [b]significantly different from diabetic control.

Pioglitazone may increase the risk of bladder cancer, according to health warnings issued by the European Medicines Agency (EMA), Healthcare Products Regulatory Agency (MHRA), and Food and Drug Administration (FDA)24. In contrast, Wei et al[25] found no significant correlation between pioglitazone usage and bladder cancer risk in a large primary care sample of UK type 2 diabetics. Pioglitazone causes weight gain, pedal oedema, bone loss, and congestive heart failure in at-risk patients, but not cardiovascular disease or all-cause mortality.

In this study, STZ was used to induce DM in rats as accepted DM animal model as it caused β-cell damage, the main event in DM development[26]. The current study demonstrated that STZ-induced DM and IR, resulting in hyperinsulinemia, hyperglycemia and increased HbA1c levels. In contrast, DM rats treated with PGZ+RVT combined resulted in improved glycemic control and insulin levels with values closed to control rats.

These results were similar to other reported findings[27]. In DM animal's model, RVT is one of the important herbs confirmed to exhibit anti-DM effects. It results to increase glucose uptake by various cells by stimulating intracellular glucose transport[28]. This RVT effect was also reported in absence of insulin[29]. Several research[30] results have shown that RVT enhance insulin sensitivity in experimentally induced IR animal model. Beside animal's models, human clinical studies also using commercial oral hypoglycemic agents along with RVT showed its synergistic effect on HbA1c improvement and glycemic control[31].

Similar to previous studies[22], this study clearly confirmed the presence of IR in STZ-induced DM by increased HOMA-IR. Administration of PGZ+RVT combined attenuated the induced IR in DM animals. These findings confirm that the synergistic effect of RVT and PGZ against DM-induced hepatic IR and β-cell function enhancement.

Supporting its anti-diabetic actions, RVT has been demonstrated to ameliorate hyperglycaemia-mediated disturbances in lipid levels in rats and its related inhibition of formation of atherosclerosis[32,33]. Here, RVT, when used in combination with PGZ, had synergistic effect for effecting beneficial changes in cholesterol, triglycerides, LDL and HDL levels. It also improved related atherogenic biomarkers including AIP, AC and CRR. Many pathways may be associated with RVT anti-atherogenic effect[34].

Regarding DM pathogenesis in human and animal models, many studies elucidate oxidative stress implication as common factor that causes increasing tissues specific IR[26]. Also in tissues, hyperglycemia can markedly increase the production of oxidative stress and can also enhance the imbalance between anti-oxidative protective system and reactive oxygen species (ROS) production33. Moreover, association of inflammation and DM is an active research item. Inflammation role was previously demonstrated in the DM development and hyperglycemia was reported to elevate the circulating inflammatory markers[35]. Recent studies[26] on animal and human models exhibit that inflammation and IR are associated directly with each other throughout DM development. Thus, it is important to discover therapies against oxidative and inflammatory stresses as standard treatments for DM[26].

In DM, antioxidant and anti-cytotoxic effects of RVT have been suggested to play an important role in protecting the pancreas[21]. In this study, treatments with PGZ+RVT attenuated the increase in inflammatory parameters (CRP, TNF-α and IL-6) and decreased TAC and PPARϒ levels that induced by STZ. RVT antioxidant activity depends on its functional groups' arrangement. Substitution, configuration, and total hydroxyl groups number stimulate many antioxidant activity mechanisms, such as metal ion chelation and radical scavenging abilities36. In aqueous simulated media, study of RVT antioxidant effect against hydroperoxyl (•OOH) and hydroxyl (•OH) radicals revealed that it may act as an efficient radicals scavenger[37]. Studies reported[38] RVT ability to reduce inflammatory factors expression and secretion. RVT anti-inflammatory activity may prevents inflammation by inhibiting TNF-α and IL-6 serum levels, cyclooxygenase-2 activity and macrophage inflammatory protein-2 levels, caspase-3/9 and reactive oxygen species production[37].

Conclusions

This study highlighted the synergistic effect and anti-diabetic potential of PGZ and RVT combined therapy. This treatment can delay DM onset and subsequently minimizes the risk of its related complications. This may be through insulin sensitivity improvement and free radical scavenging. This treatment attenuated lipid accumulation caused by elevated glucose levels. Moreover, it alleviated pro-inflammatory cytokines expression and restoring the antioxidant capacity.

Ethics Approval
All experiments and animals involved in this study were carried out according to the National Research Council's suggested criteria for animal care and use. The study protocol was approved by the Animal Ethics Committee of School of Graduate Studies, Jordan University.

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The authors declare they have no financial interests.

Data Availability
The data presented in this study are available on request from the corresponding author.

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Conflict of Interest
Authors have no conflicts of interest to declare.

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