Efficacy of metformin on different adipocytokines in type 2 diabetes mellitus patients

Naglaa Hamdy[a], Mohamed Abdel-Gabbar[a], Hader I. Sakr[b,c], Mohamed A. Abdelaziz[d], Mohamed Kandeil[e], Ayman M.Abdel Aziz[f], Osama M. Ahmed[g]

[a]Department of Biochemistry, [g]Physiology Division, Department of Zoology, Faculty of Science, Beni-Suef University, Beni-Suef P.O. Box 62521, Egypt, [e]Department of Biochemistry, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt, [b]Department of Medical Physiology, Faculty of Medicine, Cairo University, Cairo 35855, Egypt, [f]Cell Biology, Histology and Genetics Division, Department of Zoology, Faculty of Science, Fayoum University, Fayoum, Egypt, [c]Department of Medical Physiology, General Medicine Practice Program, Batterjee Medical College, Jeddah, Saudi Arabia, [d]Department of Basic Medical Sciences, College of Medicine, Prince Sattam Bin Abdulaziz University, Alkharj 11942, Kingdom of Saudi Arabia

Correspondence to Hader I. Sakr, MD, PhD, Department of Medical Physiology, Faculty of Medicine, Cairo University, Cairo 35855, Egypt
Tel: +20 111 161 6364; fax: +22 727 2148;
e-mail: hadersakr@kasralainy.edu.eg

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Background
Type 2 diabetes mellitus is a major medical condition that constitutes a significant financial burden on most healthcare systems.
Objective
The current research aimed to evaluate the antidiabetic, anti-inflammatory, and antihyperlipidemic effects of 500 mg metformin twice daily for 6 months on various adipocytokines in type 2 diabetes mellitus patients.
Patients and methods
The participants in this study were divided into three groups: the control, the untreated diabetic, and the metformin-treated diabetic groups.
Results and conclusion
Metformin treatment significantly improved the poor oral glucose tolerance and the lowered serum levels of insulin and C-peptide with subsequent better homeostatic model assessment for insulin resistance and sensitivity and β-cell function results. Moreover, metformin treatment significantly decreased the elevated serum levels of glycosylated hemoglobin, high, low, and very low-density lipoproteins, adipokines (visfatin and resistin), and retinol-binding protein-4 expression, with a significant increase in total cholesterol and triglycerides. Metformin also reduced the proinflammatory cytokine expressions (interleukin-1β, interferon-γ, and tumor-necrosis factor-α). In conclusion, metformin can alleviate adipocytokines through anti-inflammatory effects, synergizing with its antidiabetic actions.

Keywords:
adipokines, cytokines, insulin resistance, lipid profile, metformin, type 2 diabetes mellitus

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Introduction

Besides being a chronic metabolic and endocrine condition, diabetes mellitus (DM) is marked by elevated blood glucose levels that induce many significant issues [1]. It is characterized by abnormal metabolism of carbohydrates, lipid [2], and protein [3]. Type 2 DM (T2DM) has numerous serious complications, such as retinopathy, cardiovascular disease, nephropathy, and neuropathy, and is considered a significant global public health problem [4]. A well-known and controllable risk factor for cardiovascular disease is diabetic dyslipidemia [5]. As a result, controlling diabetic dyslipidemia has been identified as an essential part of a multifactor approach to avoid cardiovascular disease in T2DM patients [6]. Elevated blood low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglycerides (TG) values are the most serious problems linked with dyslipidemia. So, the efficacy of antihyperlipidemic activity is generally based on decreasing these serum levels [2].

Adipose tissue was traditionally considered an energy storage depot with few exciting properties. Still, it is now recognized as an active endocrine organ that secretes many bioactive mediators called adipokines. These adipokines regulate blood pressure, inflammation, lipid and glucose metabolism, fat mass, and nutrient homeostasis [7]. The vital activities of adipokines regarding satiety, appetite, glucose tolerance, insulin resistance (IR), and management of fat and energy stores consolidate adipose tissue's involvement in the initiation and pathogenesis of DM and its complications [8].

Several adipokines, including tumor necrosis factor-alpha (TNF-α), visfatin, resistin, and retinol-binding protein 4 (RBP4), aroused the curiosity of researchers. In addition, proinflammatory mediators from the

adipose tissue are hypothesized to contribute to vascular damage, IR, and cardiovascular diseases [9,10]. These adipokines have either anti-inflammatory or proinflammatory effects with IR properties [11].

Visfatin is an endocrine, autocrine, and paracrine peptide that can alter insulin sensitivity (IS) with its insulin receptor activator effect [12]. Moreover, resistin inhibits glucose uptake and promotes IR, modulating fasting blood glucose (FBG) levels. Resistin and visfatin are upregulated in T2DM. These adipocytokines can also predict cardiac diseases in diabetic patients because they promote vascular dysfunction. Resistin and visfatin levels increase with obesity [13]. RBP4 is another potential diabesity target that transports plasma retinoids [14] and is linked to IR. IS is increased by RBP4 genetic mutation [15]. Macrophages have a significant role in accelerating inflammation, IR, and T2DM during obesity [16].

Biguanide metformin hydrochloride is the most widely used oral hypoglycemic due to its effectiveness, low price, weight neutrality, and good safety record [17,18]. Due to its ability to lower blood glucose and glycosylated hemoglobin (HbA1c) levels, metformin is used alone or in combination with other antihyperglycemic medications as the first-line oral therapy [19]. Metformin exerts its primary effects by reducing hepatic glucose production. Whether metformin has an insulin-sensitizing effect in peripheral tissues remains controversial [20].

Herein, the study scrutinized the changes in adipokines and proinflammatory cytokines resulting from metformin treatment in type 2 diabetic patients. Also, the antidiabetic protective characteristics of metformin on T2DM were examined.

Patients and methods

The study's target population is T2DM patients treated with metformin or who did not take antidiabetic drugs. The samples were collected at zero-time (baseline) and after 6 months of treatment from the three equal groups (n=30).

The control group: included healthy individuals.

The untreated diabetic group: newly diagnosed and previously untreated T2DM patients.

The metformin-treated diabetic group: consists of diabetic patients receiving 500 mg of oral metformin twice daily.

Before enrollment, the study groups underwent careful and detailed laboratory investigations to exclude any condition that may interfere with glucose tolerance. The patients were asked to attend the clinic for follow-up every 14 days throughout the study period.

Inclusion criteria

Participants had fasting serum glucose (FSG) from 70 to 110 mg/dl, representing the normal group. Patients with FSG ranged from 125 to 200 without any diabetic treatment before, representing the untreated group. Groups were asked to prevent sugar, reduce carbohydrates, and walk for 1 h daily.

Exclusion criteria

Patients suffering from autoimmune disorders or those recently hospitalized for infection were excluded. Neither the patients nor the controls had a history of clinical or standard laboratory results diagnostic of parasitic, viral, or other infection or impaired hepatic or renal function. Pregnant or lactating women, patients with other medical conditions other than T2DM, and smokers were excluded from the study. Any newly diagnosed diabetic patient (untreated group) with a FSG of more than 200 mg/dl was excluded. Patients who did not follow the treatment, diet, or exercise or missed the follow-up two consecutive times were excluded. Any patient in the metformin-treated diabetic group who changed metformin treatment through the 6 months was excluded.

Ethical approval and study design

In this study, blood samples were collected from El-Wasta Central Hospital, Beni-Suef Governorate, Egypt. They were obtained after approval of the volunteers, the diabetic patients, and the hospital administration. Fayoum University Supreme Committee for Scientific Research Ethics (FU-SCSRE) approved the study (Code No. of the proposal: EC 2210 and final approval date: 31/01/2023). All authors accept to transmit the authors' right to the publishing journal upon acceptance.

Body weight and blood sampling

Weight and height were measured at the zero time and after 6 months. An automatic electronic balance (GRANZIA SRLS Instrument, via Sant anna 1, Genova, Italy) was used to measure changes in body weight. BMI was calculated [21], and medical history, medications, and daily diet were recorded.

After getting a brief history from all participants, 10 ml morning venous blood samples were taken after an overnight fast of 6–8 h. The collected blood was

subdivided as follows: 4 ml was allowed to clot in plastic Hoffman tubes for the determination of insulin, C-peptide, and adipokines using enzyme-linked immunosorbent assay (ELISA); 1 ml was collected and gently thoroughly mixed in fluoride tubes to measure the level of glucose concentration; 3 ml blood was gathered in EDTA tubes, and thoroughly mixed for use in molecular detection of inflammatory mediators by real-time PCR technique, and 2 ml was collected in EDTA tubes for measuring HbA1c. Then, all participants were asked to continue fasting for another 4 h and 5 ml venous samples was collected again for lipid profile biochemical analysis. All tubes were centrifuged at 4000 rpm. for 15 min to obtain a separate serum. The serum was either examined instantly or kept at −20°C for subsequent analysis.

Biochemical investigations

Using a reagent kit obtained from BioSystems, Maadi, Cairo, Egypt, serum fasting glucose was measured using glucose oxidase and 4-amino antipyrine [22].

Using a BIOS Human INS ELISA kit, serum fasting insulin (FI) was measured by the quantitative enzyme-linked immunoassay method (Catalog Number is 10801) purchased from Chemux Bio Science Inc. (South San Francisco, California, USA). Homeostasis Model Assessment (HOMA) was calculated based on FI and FBG levels. HOMA-IR method, HOMA β-cell function (HOMA-β), and HOMA-IS were estimated as follows: [23]

$$\text{HOMA} - \text{IR} = (\text{FBG in mg/dl}) \text{x FI in} \mu\text{IU/ml})/405$$

$$\text{HOMA} - \beta\text{cell function} = (20 \text{ x FI in} \mu\text{IU/ml})/[(\text{FBG in mg/dl} \div 18)3.5]$$

$$\text{HOMA} - \text{IS} = 10000/(\text{FI in mIU/ml x FBG in mg/dl})$$

Assessment of C-peptide was done using BIOS Human ELISA kit (Catalog Number is 10802) purchased from Chemux BioScience Inc, as instructed by the manufacturer. Quantitative determination of HbA1c was performed using a reagent kit bought from Egypt's BioMed [24].

RNA extraction

Using a Trizol/tri kit purchased from Qiagen Inc. (South San Francisco, California, USA), total RNA was isolated from whole blood [25]. A reagent kit from Applied Biosystems (California, USA), synthesized cloned DNA (cDNA). The total RNA concentration was evaluated using a Nanodrop 2000 spectrophotometer. The ratio of the extracted RNA's integrity (A260/280) was ~1.8, and the total RNA used for cDNA synthesis was about 10 µl of total RNA.

Expression of proinflammatory cytokines

The mRNA expression of the proinflammatory cytokines [interleukin-1 beta (IL-1β), TNF-α] [26] and gamma interferon (IFN-γ) [27] was detected using the RT-PCR technique. RNA purification kit was purchased from Qiagen Inc. (Germantown, USA) (Catalog Number is 52906), and primers were purchased from Bioresearch Technologies (Petaluma, California, USA).

The following primers were used:

IL-1β forward 5′-AAACAGATGAAGTGCTC CTTCCAGG-3′ and reverse 5′-CTCCTTAATGT CACGCACGATTTC-3′

TNF-α forward 5′-TCTCGAACCCCGAGTGA CAA-3′ and reverse 5′-TATCTCTCAGCTCC ACACCA-3′

IFN-γ forward 5′-GCATCGTTTTGGGTTCTC TTGGCTGTTACTGC-3′ and reverse 5′-CTCCT TTTTCGCTTCCCTGTTTTAGCTGCTGG-3′

Expression of adipokines

Quantitative determination of the adipocytokines visfatin [28], resistin, and RBP4 [29] were determined using ELISA kits from RayBio (Norcross, Georgia, USA) (Catalog Number EIA-VIS and EIA-RES and EIA-RBP, respectively).

Lipid profile

Serum lipids detection kits for TC and TG were purchased from DiaSys Diagnostic Systems (GmbH, Germany). The high-density lipoprotein cholesterol [30] was purchased from Spinreact (Spain). LDL-C and very low-density lipoprotein-cholesterol were calculated as follows [31,32]:

$$\text{VLDL} - \text{C} = \text{TG}/5$$

$$\text{LDL} - \text{C} = \text{TCHDL} - \text{C}(\text{TG}/5)$$

Statistical analysis

The IBM Statistical Program for the Social Sciences (SPSS Inc., Chicago, IL, USA), version 22.0 was applied to analyze the results in the present study. Data were analyzed using one-way analysis of variance and Duncan's test post-hoc analysis. Results were presented as median±SD. *P* values more than 0.05 were insignificant, while *P* value less than 0.05 were significantly different.

Results

Demographic data

The demographic representation of data is demonstrated in Table 1. The biochemical

Table 1 Demographic data of the studied groups

	Groups		
Variables	Control	Untreated diabetic	Glibenclamide-treated diabetic
Number of patients	30	30	30
	Age (years)		
Mean±SE	50.11±2.19	49.95±2.04	48.50±0.86
	Sex [*n* (%)]		
Male	6 (20)	12 (40)	6 (20)
Female	24 (80)	18 (60)	24 (80)

Table 2 Comparison of visfatin, resistin, and retinol-binding protein 4 concentrations among the study groups at zero time and after 6 months

Variables	Control group	Untreated diabetic group	Metformin-treated group	*P*
		Visfatin (ng/ml)		
Zero time	0.84±0.008 [b]	6.82±0.24 [e]	5.76±0.27 [d]	<0.05
6 months	0.82±0.012 [a]	8.98±0.35[f]	2.30±0.12 [c]	<0.05
		Resistin (ng/ml)		
Zero time	6.25±0.13 [b]	10.8±0.40 [e]	9.95±0.39 [d]	<0.05
6 months	6.11±0.13 [a]	18.56±0.65[f]	6.75±0.28 [c]	<0.05
		RBP4 (ng/ml)		
Zero time	6.35±0.15 [b]	18.96±0.76 [e]	15.91±0.51 [d]	<0.05
6 months	6.27±0.18 [a]	22.04±0.89[f]	10.89±0.47 [c]	<0.05

Data are presented as mean±SE.
RBP4, retinol-binding protein 4.
Different superscript letters are considered significantly different for each parameter at *P* value less than 0.05.

evaluation of the studied groups is shown in Table 2. We noticed that the BMI decreased in all groups after 6 months compared with the zero-time; however, this decrease was only statistically significant ($P<0.05$) in the diabetic untreated group. The values of FBG and HbA1c after 6 months were statistically significantly ($P<0.05$) increased in the control and untreated diabetic groups and significantly ($P<0.05$) decreased in the metformin-treated diabetic group compared with their corresponding values at zero time. The HOMA-IR values after 6 months were statistically significantly ($P<0.05$) decreased in the diabetic groups and statistically nonsignificantly ($P>0.05$) changed in the control group compared with their corresponding values at zero time. Moreover, the values of FBG, HbA1c, and HOMA-IR after 6 months were statistically significantly ($P<0.05$) increased in the two diabetic groups compared with the control group and statistically significantly ($P<0.05$) decreased in the metformin-treated diabetic group compared with the untreated diabetic group.

Concerning the values of serum C-peptide, FI, and the calculated HOMA-β cell functions and HOMA-IS after 6 months, they were generally decreased in the control and untreated diabetic groups and increased in the metformin-treated diabetic group compared with their corresponding values at zero time. Such changes were only statistically significant ($P<0.05$) in the case of the control group's C-peptide, the untreated diabetic group's FI and HOMA-β cell functions, and the metformin-treated diabetic group's HOMA-β cell functions and HOMA-IS. Also, the 6- month values of the two diabetic groups' serum C-peptide and FI were statistically significantly ($P<0.05$) increased, and that of the calculated HOMA-β cell functions and HOMA-IS were statistically significantly ($P<0.05$) decreased compared with the control group, except the metformin-treated diabetic group's value of the calculated HOMA-β cell functions that became statistically nonsignificant ($P>0.05$). Nevertheless, the values of serum C-peptide and FI in the metformin-treated diabetic group after 6 months were statistically significantly ($P<0.05$) decreased compared with the untreated diabetic group.

Changes in lipid profile

As shown in Table 2, the results of the present study showed statistically nonsignificant ($P>0.05$) changes in the mean values of high-density lipoprotein cholesterol and very low-density lipoprotein-cholesterol after 6 months among the different groups compared with each other and compared with their corresponding values at zero time. In contrast, the mean values of TC, LDL-C, and TG after 6 months showed a statistically significant

(P<0.05) decrease in the three groups (except the TG in the control group) compared with their corresponding values at zero time. The mean values of TC, LDL-C, and TG after 6 months also showed a statistically significant (P<0.05) increase in the diabetic groups compared with the control group (except the TG in the metformin-treated diabetic group) and a statistically significant (P<0.05) decrease in the metformin-treated diabetic group compared with the untreated diabetic group.

Changes in serum levels of visfatin, resistin, and retinol-binding protein 4

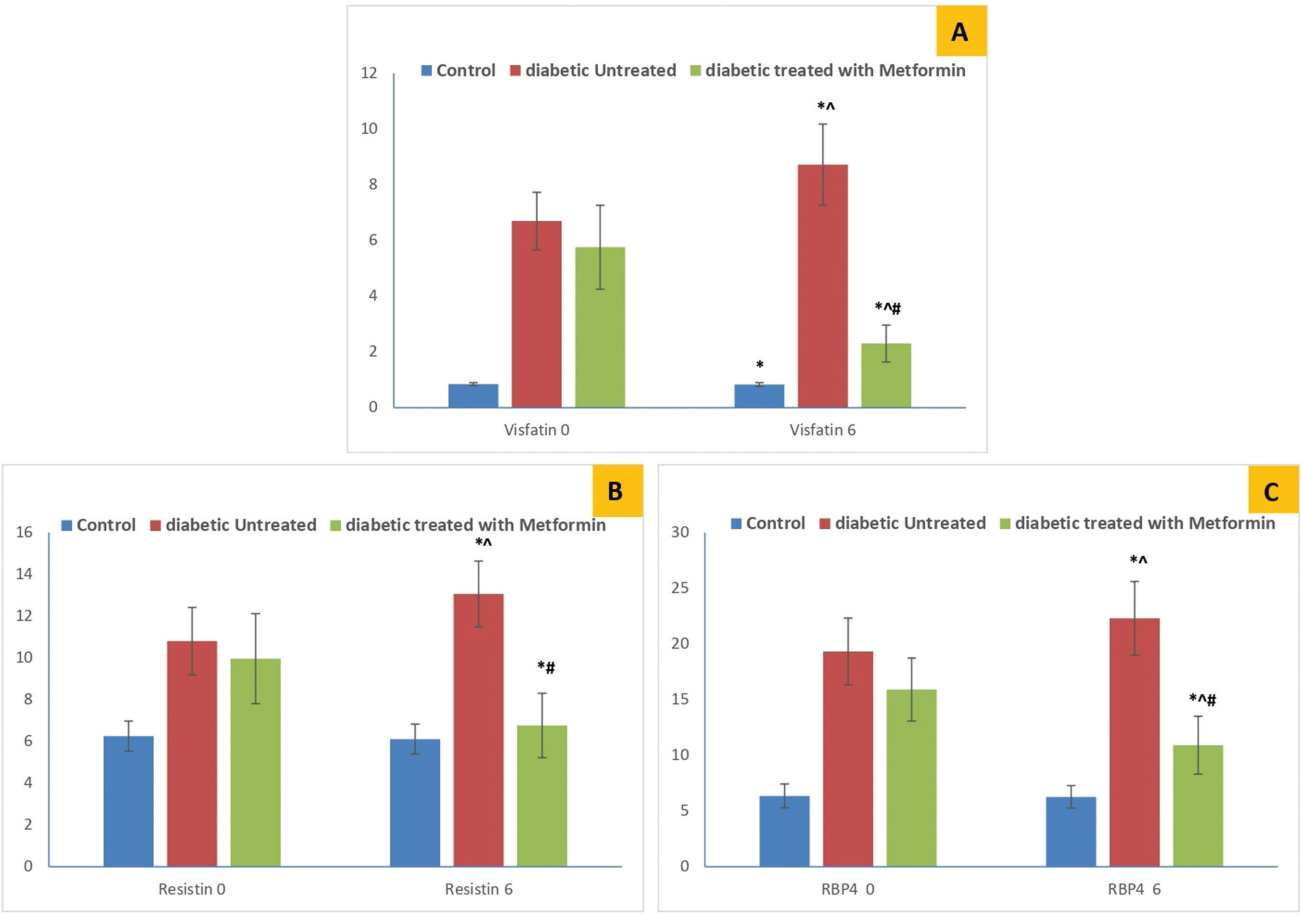
As shown in Fig. 1, serum visfatin, resistin, and RPB4 levels after 6 months showed a statistically significant (P<0.05) change in the diabetic groups and a statistically nonsignificant (P>0.05) decrease (except visfatin) in the control group compared with their corresponding values at zero time. Moreover, serum visfatin, resistin, and RPB4 levels after 6 months showed a statistically significant (P<0.05) increase in diabetic groups (except resistin in the metformin-treated diabetic group) compared with the control group and a statistically significant (P<0.05) decrease in the metformin-treated diabetic group compared with the untreated diabetic group.

Effect of metformin on mRNA expression of various cytokines

The expression of interleukin-1β

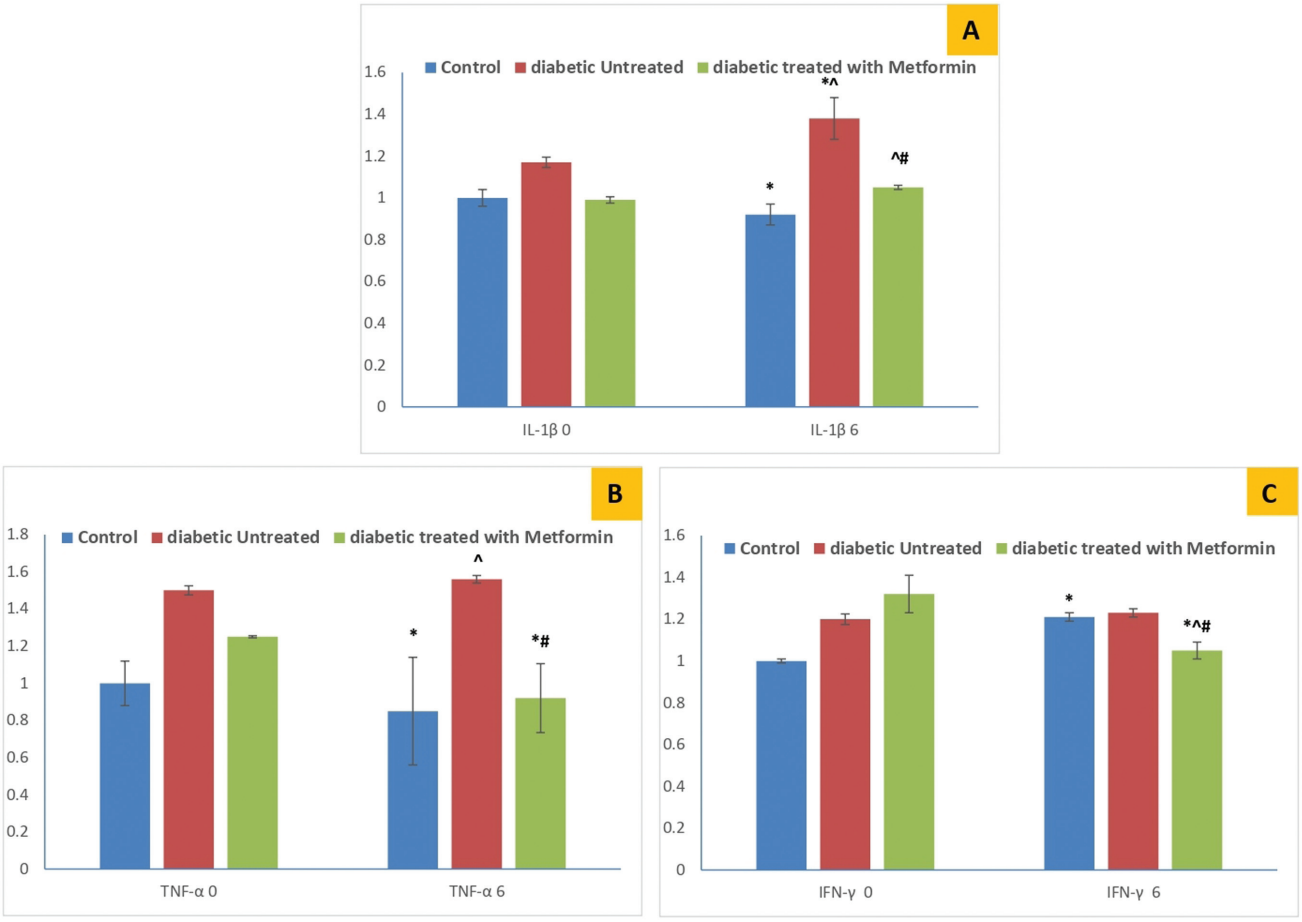
The expression of the pro-inflammatory cytokine IL-1β after 6 months showed a statistically significant (P<0.05) change in the control and untreated diabetic groups and a statistically nonsignificant (P>0.05) increase in the metformin-treated diabetic group compared with their corresponding values at zero time. Nevertheless, IL-1β expression showed a statistically significant (P<0.05) increase in the diabetic groups compared with the control group and a statistically significant (P<0.05) decrease in the metformin-treated diabetic group compared with the untreated diabetic group (Fig. 2a).

Figure 1



Comparison between (a) visfatin, (b) resistin, and (c) RBP4 concentrations among the study groups at zero time and after 6 months. RBP4, retinol-binding protein 4.

Figure 2



PCR results of (a) IL-1β, (b) TNF-α, and (c) IFN-γ concentrations among the study groups at zero time and after 6 months. Data are presented as average±SD. Statistically significant ($P<0.05$) when compared with (*) the same group at zero time, (^) the control group after 6 months, and (#) the untreated diabetic group after 6 months. IFN-γ, gamma interferon; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-alpha.

The expression of tumor necrosis factor-alpha and gamma interferon

The expression of proinflammatory cytokines TNF-α and IFN-γ after 6 months showed a statistically significant ($P<0.05$) change in the control and metformin-treated diabetic groups and a statistically nonsignificant ($P>0.05$) increase in the untreated diabetic group compared with their corresponding values at zero time. Their expression after 6 months also demonstrated a statistically significant ($P<0.05$) decrease in the metformin-treated diabetic group compared with the untreated diabetic group. The 6-month expression of TNF-α in the untreated diabetic group and IFN-γ in the metformin-treated diabetic group showed a statistically significant ($P<0.05$) change compared with the control group (Fig. 2b, c).

Discussion

DM is a metabolic disease brought on by impaired glucose metabolism. It is characterized by high blood glucose and insulin levels and inadequate insulin response in cells [33]. Metformin is a biguanide derivative (1,1-dimethyl biguanide hydrochloride) derived from *Galega officinalis* [34]. Metformin can improve the glycemic profile and reduce cardiovascular mortality without the risks of hypoglycemia and/or weight gain associated with other antidiabetic medications [35]. Metformin is also the preferred antidiabetic drug due to its low cost, effectiveness, and favorable safety. It does not produce clinical hypoglycemia in T2DM patients or change glucose homeostasis in nondiabetic people [36,37]. Despite numerous research studies to better understand metformin's potential in T2DM patients, several aspects of its mode of action are still unclear.

Treatment with metformin improves HbA1c, IR, and endothelial function in T2DM [38]. In the same regard, metformin reduces hepatic glucose production and enhances muscle glucose uptake associated with improved IR [39,40]. In agreement, this study showed improved IR, increased serum FI, HOMA-β cell function, and IS with a concomitant

decrease in serum levels of FBG, HbA1c, and HOMA-IR in T2DM treated with metformin for the 6 months. The improved β-cell function could explain the metformin-induced improvement in IS. C-peptide is produced at equal levels as insulin, and according to Maddaloni *et al.* [41], it is the most accurate indicator of endogenous insulin production in diabetic patients. Our results showed a lower C-peptide after 6 months of metformin treatment than the untreated diabetic group.

Gillani *et al.* [42] and Chaudhury *et al.* [43] demonstrated decreased serum lipid profile levels in T2DM with oral metformin, suggesting an antihyperlipidemic activity. The present study showed a significant increase in serum TC, TG, and LDL-C levels in untreated T2DM patients and a significant decrease in their levels in diabetic patients treated with metformin for 6 months.

Visfatin is an inflammatory mediator increased in obesity, IR, T2DM, and proinflammatory states. Compared with the controls, patients with newly diagnosed diabetes or untreated diabetics had considerably higher serum levels of visfatin [44]. In contrast, Zhang *et al.* [45] stated that serum visfatin levels significantly correlated with visceral fat but not subcutaneous fat. As a result, obesity seems to be the primary cause of an increase in T2DM. Obese participants had decreased plasma levels of visfatin considerably. The current investigation revealed a significant decrease in serum visfatin levels in T2DM treated with metformin and a significant increase in untreated T2DM compared with controls.

Resistin promotes cardiovascular disease through proinflammatory pathways [45]. Obese people and T2DM patients have higher resistin levels in their serum [13]. Serum resistin levels were positively correlated with HOMA-IR, TNF-α, and IL-6 in the T2DM group. A previous publication provides evidence that endothelial dysfunction and inflammation play a role in T2DM's resistin-linked IR [45]. It is important to note that serum resistin levels increased significantly in untreated diabetic patients and decreased significantly after 6 months in patients treated with metformin.RBP4 is an adipocytokine related to IR and IS. RBP4 is another potential diabesity target that transports plasma retinoids [46]. The present study results reported a significant increase in serum levels of RBP4 in untreated diabetic patients and T2DM treated with metformin compared with controls after 6 months. Metformin treatment for 6 months significantly decreased serum levels of RBP4 compared with zero time.

Most often, macrophages are blamed for developing inflammation during obesity, triggering IR and eventually T2DM [47]. Metformin administration improves IS by inhibiting the synthesis of nitric oxide, prostaglandin E, and proinflammatory cytokines (IL-6 and TNF-α) and limiting the expression of the major transcription factor responsible for inflammatory response, the nuclear factor NF-KB [48]. According to clinical research, 3-month metformin treatment in patients with hypertension and type 2 diabetes reduces HbA1c, IL-8, IR, systemic inflammation [49], and TNF-α [50].

So far, no treatment has eliminated DM and its complications [51]. When using an overdose of metformin, more than 30% of T2DM patients experience side effects such as diarrhea, vomiting, anorexia, stomach pain, nausea, flatulence, and drowsiness. Also, long-term use of metformin decreases gastrointestinal absorption of vitamin $B_{12,}$ resulting in severe anemia [52].

Limitations of the study

This study was relatively short term and mostly looked at parameters of glycemic efficacy. The long-term microvascular and macrovascular effects have not been thoroughly investigated. Large sample size prospective studies are required to demonstrate the function of adipokines in early IR prediction in populations at increased risk of developing T2DM. The pathogenic significance of adipocytokines and cytokines in endothelial dysfunction and inflammation must be further studied.

Metformin is considered the first-line pharmacotherapy used in the management of T2DM. However, both sulfonylurea and biguanide antihyperglycemic agents are the most common and widely distributed oral hypoglycemic. A larger number of cases is required for detecting their effects in normal populations and comparing their effects, for more confirmation and validation of our results that are of significance in detecting different aspects of metformin action in T2DM cases.

Conclusion

Based on our results, we can conclude that metformin has an anti-inflammatory potency in treating patients with T2DM through its modulatory effects on the

adipocytokines and the proinflammatory cytokines IFN-γ and TNF-α.

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Naglaa Hamdy[a], Mohamed Abdel-Gabbar[a], Hader I. Sakr[b,c], Mohamed A. Abdel Aziz[d], Mohamed Kandeil[e], Ayman M. Abdel Aziz[f], and Osama M. Ahmed[g] Research conception and design: N.H., M. A.G., M.K., and O.M.A.; experiments: N.H., M.A. G., M.K., A.M.A.A., and O.M.A.; statistical analysis of data: H.I.S., M.A.A., and O.M.A.; interpretation of the data and writing of the manuscript: all authors; work revision and final approval: H.I.S., M.A.A., and O.M.A.

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