Study on the potential antidiabetic effects of trigonelline alone and with vildagliptin in type-2 diabetic model in rats
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Abstract

Trigonelline is the major alkaloid component of fenugreek that proved antidiabetic efficacy; however its mechanism of action has not yet been elucidated. The objective of this work was to determine the effects of trigonelline and vildagliptin on the altered insulin signaling, glucose hemostasis, and lipid profile in insulin resistance /type 2 diabetic model in rats induced by high-fat/fructose diet (HFFD) for 8 weeks followed by a single sub-diabetogenic dose of streptozotocin (35 mg/kg, i.p.). Trigonelline (50mg/kg, p.o, daily for 4 weeks) decreased body weight, visceral and epididymal fat weight ratios. Moreover, it opposed the HFFD effects lowering serum levels of glucose, insulin, and fructosamine levels, as well as the homeostatic model assessment–insulin resistance (HOMA-IR) index. Besides, it improved serum total cholesterol and triglycerides, as well as serum alanine and aspartate aminotransferases. In the soleus skeletal muscle, trigonelline elevated insulin receptors autophosphorylation, protein kinase B, glucose transporter 4 (GLUT4), and glutathione. In addition, it decreased soleus skeletal muscle advanced glycation end products (AGE), free fatty acids (FFAs), triglycerides, and lipid peroxides. Likewise, diabetic groups treated with both doses of vildagliptin (3 and 10mg/kg, p.o, daily for 4 weeks) showed noticeable improvements in the aforesaid parameters. In conclusion, the present study demonstrates that trigonelline significantly improved glucose and lipid homeostasis in insulin resistant/diabetic animals by improving insulin sensitivity via enhanced GLUT4 and the inhibition of FFAs, and AGE as well as its antioxidant potential in the soleus skeletal muscle.

Keywords: Trigonelline, Vildagliptin, Insulin resistance, HFFD, GLUT4, FFA
INTRODUCTION

Diabetes mellitus (DM) is one of the most widespread endocrine chronic diseases that become epidemic in developing countries and is among the top five main causes of death in the developed ones (WHO, 2012; DeBandeira et al., 2013). The world prevalence of DM in 2014 was 8.3%, with an estimated number of 387 million carriers and is expected to reach 592 million by 2035 (IDF Diabetes Atlas, 2014). DM is a common and serious metabolic disorder of multiple etiology, characterized by chronic hyperglycemia with disturbances of carbohydrate and fat metabolism. DM results from defects in insulin secretion and/or action (Alberti and Zimmet, 1998; Pop-Busui et al., 2006).

DM complications include long-term damage, dysfunction, and failure of various organs, viz., heart, kidney, brain, and eyes (Brownlee and Cerami, 1981; Pop-Busui et al., 2006; Meetoo et al., 2007; Valko et al., 2007). The risk of developing cardiovascular diseases (CVD) augments linearly with increased glycemia (Vehkavaara et al., 1999), favoring the emergence of atherosclerosis and cardiac diseases. The relative risk of death due to vascular complications is three-fold higher in patients with DM than in the remaining population (Haffner et al., 1998; Schaan et al., 2004).

Insulin resistance (I/R)

Under normal physiological conditions, the glucose concentration remains within a narrow range in the fasting, as well as in the fed states (Bolli and Fanelli, 1999). This tight glucose regulation is maintained by a delicate balance between insulin secretion and insulin sensitivity (Kahn, 2003). In individuals with normal glucose tolerance
(NGT), a decrease in insulin sensitivity of the peripheral tissues results in a compensatory increase in insulin secretion and normoglycemia is maintained (Arslanian, 2005). However, type 2 diabetes is characterized by two major pathophysiologic defects, namely, I/R and impaired β-cell secretory function (Bloomgarden, 2007).

I/R is an impaired biological response to the effects of exogenous or endogenous insulin (Grossman, 2002). Insulin resistance in the hepatic and peripheral tissues, particularly skeletal muscle, leads to unrestrained hepatic glucose production and diminished insulin-stimulated peripheral glucose uptake and utilization (DeFronzo et al., 1992). Insulin secretion by the pancreatic β-cell is initially sufficient to compensate for insulin resistance, thereby, maintaining normal blood glucose levels (Kahn et al., 2006; Bloomgarden, 2007). However, in patients who may develop type 2 diabetes, insulin secretion eventually fails, leading to hyperglycemia and clinical diabetes (Kahn et al., 2006).

**High fat/fructose diet (HFFD)/ STZ**

The animal model described in this work simulates a typical Westernized unhealthy habit, in which HFD is combined with fructose in drinking water (20%), to produce I/R. HFFD is followed by a subdiabetogenic dose of streptozotocin (STZ) that would elevate blood glucose to the diabetic level without destroying much of the β-cells (Schaalan et al., 2009). The expected I/R syndrome is characterized by increased body weight, hypertriglyceridemia, hypercholesterolemia, compensatory hyperinsulinemia, and I/R in the peripheral tissues (Schaalan et al., 2009).

This rat model of I/R, on one hand, closely reflects the natural history and metabolic characteristics of human type 2 diabetes and, on the other hand, it is not expensive, easily accessible, practical, and can be used to test
various compounds for the treatment of type 2 DM (Zhang et al., 2008; Schaalan et al., 2009).

Although many animal models of type 2 DM have been developed, they are based on either high fat intake (HFD) or high fructose feeding. The first model induces glucose intolerance with impaired pancreatic function and hypercholesterolemia (Willett, 2002), while the second causes hypertriglyceridemia and hyperinsulinemia. In both diet regimens, the blood glucose level does not reach the diabetic levels; hence, it remains unsuitable for the validation of antidiabetic drugs, such as insulin secretagogues or insulin sensitizers (Basciano et al., 2005). Rats fed with the diet described in this work differ in the way that animals receive both high fat and high fructose in their diet. This combination synergizes their metabolic consequences, an approach that renders rats obese, insulin resistant, mild hyperglycemic, hypercholesterolemic, and hypertriglyceridemic with compensatory hyperinsulinemia. This condition is similar to prediabetic, insulin resistant state in humans. I/R is overcome normally by β-cell-induced insulin overproduction; however, over time, the conversion of prediabetes to frank hyperglycemia in patients with type 2 diabetes becomes associated with a decline in the secretory capacity of the overwhelmed pancreatic β-cells. Therefore, the evolution of frank hyperglycemia is achieved in insulin-resistant HFFD fed rats by using a single subdiabetogenic dose of STZ (Reed et al., 2000).

**Fenugreek**

The herb fenugreek, *Trigonella foenum-graecum* L., Fabaceae family, is used both in cooking and for the treatment of diabetes in many parts of the world, especially in China, India, and Middle Eastern countries.
Moreover, fenugreek seeds are effective in the prevention of retinopathy and other diabetic complications (Preet et al., 2005; Preet et al., 2006). The biological and pharmacological actions of fenugreek are attributed to the variety of its constituents, namely, steroids, such as diosgenin (Uemura et al., 2010), alkaloids, such as trigonelline (Puri et al., 2011; 2012), amino acids, such as 4-hydroxyisoleucine (Basch et al., 2003; Singh et al., 2010), saponins (Lu et al., 2008), oil (Hamden et al., 2010a), and soluble dietary fibers (Hannan et al., 2007).

**Trigonelline**

Trigonelline is an alkaloid, chiefly obtained from fenugreek and is an important bioactive marker with estrogenic, anti-diabetic, and anti-invasive properties (Allred et al., 2009; Yoshinari et al., 2009). Other plants containing trigonelline include Tung-kua-jen (Li, 1974), and Mirabilis jalapa (Walker et al., 2008). Additionally, trigonelline occurs as a minor component in wastes from industrial coffee roasting and extraction processes (Taguchi et al., 1985). Trigonelline is a metabolite of niacin in humans (Yuyama and Suzuki, 1991), which is a component of over-the-counter vitamin supplements and is used as a hypocholesterolemic and hypolipidemic agent (Physicians' Desk Reference., 1995). Moreover, trigonelline is proposed for use in humans as an effective carrier for drug delivery, e.g. phenylethylamine (Bodor and Farag, 1983), dopamine, 2,3-dideoxynucleosides (Palomino et al., 1989), and phenytoin (Pop et al., 1989) to the brain and acyclovir to the skin (Chikhale and Bodor, 1991).

**Vildagliptin**
Vildagliptin is a novel antidiabetic agent that belongs to the DPP-4 inhibitors class; it acts on the incretin system (Kleppinger and Helms, 2007), blocks GIP (Gastric inhibitory polypeptide) (Ahren and Foley, 2008) and GLP-1 degradation (Fig. I.10) (Brandt et al., 2005). DPP-4 inhibition by vildagliptin results in higher active GLP-1 concentrations and decreased glucose concentrations (Idris and Donnelly, 2007).

Vildagliptin has been extensively studied in multiple clinical studies, including various populations with type 2 DM (Keating, 2010). The drug has demonstrated efficacy when given as monotherapy or in combination with other antidiabetic drugs or insulin (Matthews et al., 2010). It can be added to metformin (Ahren et al., 2011b), being safe with respect to cardiovascular and cerebrovascular events (Schweizer et al., 2010; 2011).
AIM OF THE WORK

Type 2 diabetes mellitus (T2DM) and insulin resistance (I/R) are common and serious metabolic disorders of multiple etiology that are increasing worldwide due to sedentary lifestyle. They are characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both. High fat diet and fructose intake (HFFD) resemble the skewed style of the recent diet worldwide. HFFD appears to be at least one very important contributing factor to the epidemic obesity and I/R along with T2DM. HFFD causes weight gain, disturbed lipid profile and metabolic consequences that are linked to impaired glucose tolerance and I/R with hyperinsulinemia.

Although different types of oral hypoglycemic agents are available for the treatment of DM, there is an increasing demand to use natural anti-diabetic products for their fewer side effects. In recent years, numerous traditional medicinal plants were tested for their anti-diabetic potential in the experimental animals. Much attention has been paid to find novel type of natural anti-diabetic constituents from various medicinal plants. Among the traditional medicinal plants used in diabetes management, fenugreek has been used in folk medicine for centuries for a wide range of diseases including diabetes. Several active constituents have been isolated from fenugreek, including trigonelline, which is a major alkaloid component of fenugreek.

Though trigonelline has been documented to mediate hypoglycaemic effects via inhibiting α-glucosidase, α-amylase, maltase and lipase, as
well as its antioxidant and antihyperlipidemic capacities, yet its other mechanisms of action has not been fully elucidated.

The present study was designed to determine the effect and the possible underlying mechanisms of action of trigonelline alone or in combination with vildaglptin. The latter was chosen as the standard antidiabetic drug used herein, since trigonelline was reported to possess a DPP-4 inhibitory action. To achieve this aim, the high fat/high fructose diet (HFFD) and a sub-diabetogenic dose of STZ was adopted in this study and several parameters were determined to assess the possible mechanism(s) of the tested agents. Besides the glucose (glucose, fructosamine, insulin, HOMA-IR) and lipid (TGs, TC, FFAs, AST, ALT)-related parameters, the insulin signaling pathway (insulin receptors autophosphorylation [IR-PH], phospho protein kinase B [pAKT], glucose transporter4 [GLUT4]), in the skeletal muscle of diabetic rats was assessed. In addition, the antioxidant capacity was determined by measuring the lipid peroxide and glutathione levels, as well as the advanced glycation endproducts (AGE).
METHODS

Animals

Male Wistar rats (80–120 g; National Research Center Laboratory, Cairo, Egypt) were housed (three rats/cage), in polypropylene cages, kept on a 12 hr light/dark cycle, and constant environmental conditions. Animals were fed commercially available normal pellet and water ad libitum, prior to the dietary manipulation. Experimental design and animal handling were performed according to the guidelines of the Animal Care and Use (ILAR, 1996) and after approval of the Ethics Committee of Faculty of Pharmacy, Cairo, Egypt (Permit Number: 766).

Tested agents

Agents used in the present study, viz., vildagliptin (Novartis Pharma Company, Cairo, Egypt) and trigonelline (Fluka, Buchs, Switzerland), were freshly dissolved in distilled water immediately before use. Both vildagliptin (3 and 10 mg/kg) (Akarte et al., 2012) and trigonelline (50 mg/kg) (Zhou et al., 2013) were administered orally.

Induction of insulin-resistance (I/R) and type 2 diabetes (T2D) in rats

Rats were divided into two dietary regimen-groups, i.e. normal fat diet (NFD) and high fat diet (HFD) with fructose (20%) in drinking water, until their body weight (BW) reached 220±40 g. Normal fat diet ingredients [fat 4.59; protein 21%; fibers 4.20%; carbohydrates 60%;
vitamins and minerals mix, calcium phosphate] yielded 3150 kcal/g. This group (n=10) served as normal control. The rest of the animals were kept on HFD [5300 kcal/g; cholesterol 1%; lard 15-20%; protein 21%, fibers (4.20%), carbohydrates 60%; minerals and vitamins mix, calcium phosphate], and fructose 20% in water (HFFD) (Schaalan et al., 2009). During the 8th week after dietary manipulation, animals on HFFD received a daily single dose of Insulatard (0.5 IU/kg, i.p) to guard against streptozotocin (STZ)-induced decrease in blood insulin level and to augment the development of I/R that lasted throughout the experimental period (Chang et al., 1999; Schaalan et al., 2009). In the first day of the 9th week, and after an overnight food deprivation, HFFD rats were injected freshly prepared STZ in citrate buffer (35 mg/kg, i.p, single dose), while the NFD group received the citrate buffer only. Periodic estimation of BW and levels of fasting blood glucose (FBG), as well as serum triglycerides (TGs), total cholesterol (TC), and insulin were determined throughout the experimental period. One week after STZ administration, only rats with persistent blood glucose levels between 200-350 mg/dl, hyperinsulinemia, and hyperlipidemia were considered diabetic/insulin-resistant and were further included in the study.

Determination of intra-peritoneal glucose tolerance test (GTT) and insulin glucose tolerance test (IGTT)

One week after STZ administration, 6 hour-food-deprived diabetic and non-diabetic rats were given glucose (2 g/kg, i.p) (Schaalan et al., 2009) without (GTT) and with (IGTT) insulin injection (0.4 IU/kg, i.p). The method was referred to Levy et al. (1984) with a slight modification to suit animal’s sensitivity. Droplets of blood from the tail vein were
withdrawn (under brief ether anesthesia) every 30 minutes, and up to 120 minutes to determine the serum glucose concentrations and to confirm the I/R state.

**Experimental design**

Rats involved in the current study were assigned into six groups (n=8-10) and treated as follows for 4 weeks:

1- Group I: Animals received the vehicle (distilled water) and served as the normal control group.

Diabetic rats were allocated into 5 groups as follows:

2- Group II: Diabetic animals were given distilled water and fed normal diet during the rest of the experimental period and were tagged as diabetic control group.

3- Group III: Diabetic rats were treated with trigonelline (50 mg/kg) (*Zhou et al., 2013*).

4- Group IV: Diabetic animals were treated with vildagliptin (3 mg/kg/day) (*Akarte et al., 2012*).

5- Group V: Diabetic rats were treated with vildagliptin (10 mg/kg) (*Akarte et al., 2012*).

6- Group VI: Diabetic rats were treated with a combination of trigonelline (50 mg/kg) and vildagliptin (3 mg/kg).

**Collection of samples**

One day after the last dose of any treatment, rats were weighed and euthanized. Animals were fasted 18 hours before the time of carnage to minimize feeding-induced variations in lipid and glucose pattern. The venous blood samples were collected from the retro-orbital sinus of each
animal and blood was left in tilted tubes for 15 minutes. Tubes were then centrifuged at 3000 rpm [880 x g] for another 15 minutes to separate sera. Serum of each sample was divided into several aliquots to avoid repeated freezing and thawing, and these aliquots were stored at -20 °C until assayed later. After the collection of blood samples, all rats were sacrificed by deep ether anaesthesia. Liver, as well as visceral and epididymal fats were rapidly removed from each animal and weighed. Soleus skeletal muscle was isolated, weighed, and homogenized using glass homogenizer (IKA T10 basic, Germany) in saline to give 10% w/v homogenate. This homogenate was centrifuged at 4000 rpm [1565 x g] for 5 minutes and the resultant supernatants were stored at -80 °C until analysis.
Results of the current study are summarized as follows:
1. The consumption of HFFD caused an elevation in body weight, as well as visceral epididymal fat indecies.
2. The model augmented the fasting serum levels of glucose, fructosamine, insulin, and HOMA-IR, while it waned insulin sensitivity.
3. In addition, the diet elevated the levels of serum TGs, TC, AST, and ALT indicating alteration in lipid profile.
4. The muscle contents of TGs, MDA, FFA, and AGEs were increased, while GSH, GLUT4, pAKT, IR-PH were reduced.

The effect of trigonelline
1- Trigonelline treatment revealed a decline in body weight gain, as well as visceral and epididymal fat indecies.
2- Trigonelline improved blood glucose level and serum fructosamine level. The latter effect was better than vildaglptin.
3- The alkaloid decreased insulin level and HOMA-IR and improved insulin sensitivity.
4- The effect of trigonelline also modulated HFFD-induced changes in the lipid profile, including TGs, TC in serum, FFAs and TGs in soleus skeletal muscle, in addition to serum ALT and AST. Trigonelline improved the pan dyslipidemia observed in the diabetic rats and its action overrid that of vildaglptin.
5- Trigonelline antioxidant effect was reflected on the replenishment of soleus skeletal muscle GSH level, besides the decrease of MDA and AGEs.
6- Trigonelline improved the insulin signaling pathway in the muscle by activating IR-PH,pAKT, as well as GLUT4.
The effect of vildagliptin

1- Treatment with vildagliptin diminished epididymal and visceral fat index, but did not affect body weight gain.
2- Vildagliptin reduced serum levels of glucose, fructosamine, and insulin, which resulted in a decrease in HOMA-IR and an improve in insulin sensitivity.
3- Vildagliptin evoked a marked reduction in lipid profile, including TGs, TC in serum, FFAs and TGs in soleus skeletal muscle, in addition to serum ALT and AST.
4- Vildagliptin decreased soleus skeletal muscle MDA and AGEs, while the GSH content was elevated compared to diabetic rats.
5- Vildagliptin improved insulin signaling pathway in the soleus skeletal muscle by activating pAKT, IR-PH, as well as GLUT4. The effect of vildagliptin on these parameters was better than that of trigonelline.

Effect of combination of trigonelline and vildagliptin

1- Addition of vildagliptin did not reflect a better effect over that mediated by trigonelline alone on body weight, visceral fat weight, epididymal fat weight, glucose, TGs, AST, and ALT in the serum, as well as AGEs, MDA, GSH in muscle.
2- Addition of vildagliptin reflected a better effect over that mediated by trigonelline alone on TC, insulin, pAKT, IR-PH and GLUT4.
3- However, the effect of the combination on pAKT, insulin, and GLUT4 was less than the effect of the vildagliptin alone.
4- Moreover, the effect of the combination on FFAs and fructosamine was less than that of trigonelline alone.
In conclusion trigonelline has antidiabetic and antihyperlipidemic effects. It normalized the altered lipid profile and decreased blood glucose and I/R partly via improving the insulin signaling pathway.