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Original Research

Whey protein upregulates muscle insulin receptor tyrosine kinase and is comparable to vildagliptin as insulin-sensitizer

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Abstract

Background and Aims: Whey protein is a natural product with an anti-hyperglycemic effect. This study aimed to evaluate the possible therapeutic effect of different concentration of whey protein compared to vildagliptin on Type 2 diabetes mellitus (T2DM) rat model and to clarify the underlying molecular mechanisms. **Material and Method:** Sixty male Wistar rats were divided into six groups: normal control, diabetes control, vildagliptin treated diabetic group, whey protein 10%, 20%, and 40% treated groups. **Results:** each of vildagliptin and whey protein exhibited anti-hyperglycemic and insulinotropic effect in diabetic rats; however the effects obtained by 40% whey protein was comparable to that of vildagliptin. The biochemical results were supported by the histopathological finding which showed significant increase in the number of β-cells in both vildagliptin and 40% whey protein versus the control group. Interestingly, 40% Whey protein was superior to vildagliptin in increasing the level of muscle insulin receptor tyrosine kinase (IRTK) thereby increasing sensitivity to insulin. **Conclusion:** the anti-hyperglycemic effect of whey protein was concentration-dependent and mediated by increasing intestinal incretin hormones, muscle (IRTK), and number of β-cells. 40% whey protein was comparable to vildagliptin as antihyperglycemic drug proved to have an insulin-sensitizing effect. It could be used safely as a substitute for diabetic patients.

Keywords: Incretin, incretin hormones, muscle; Type 2 diabetes mellitus.

Background and Aims

Type 2 diabetes mellitus (T2DM) is considered one of the most popular metabolic disorders. T2DM can contribute to early mortality and micro- and macro-vascular complications, including stroke, myocardial infarction, and loss of vision [1].

The decline of β-cell function in T2DM has been associated with the pathophysiological changes of impaired action of incretin hormones, which are secreted from the intestine in response to glucose intake [2]. Incretin hormones include glucose-dependent insulinotropic polypeptide (GIP) and glucagon like peptide-1 (GLP-1) which are responsible for stimulation of insulin secretion after glucose ingestion andinhibits glucagon release [3].

Dipeptidyl peptidase-4 (DPP4) inhibitors are an oral hypoglycemic agent. DPP4 inhibitors stimulate insulin secretion and inhibit glucagon release by elevating endogenous GLP-1 and GIP levels without risk of hypoglycemia [4]. Besides, DPP4 inhibitors improve pancreatic β-cell function by stimulating the proliferation of β-cells and inhibition of apoptosis [5]. Currently, many DPP4 inhibitors are approved for the treatment of T2DM, including saxagliptin, sitagliptin, and vildagliptin [6].

Vildagliptin elevates GIP and GLP-1 by inhibiting DPP-4, leading to improve in both fasting blood glucose (FBG) and postprandial

blood glucose (PBG) [4]. Vildagliptin increases insulin secretion and pancreatic insulin stores. Moreover, vildagliptin can increase β-cell mass and ratio [7]. On the other hand, vildagliptin has some adverse effects, including headache, cough, nasopharyngitis, fever, constipation, and gastrointestinal discomfort [8, 9].

Whey protein has insulinogenic properties as it contains a high level of branched-chain amino acids especially leucine, which are associated with the glucoregulatory effect [10]. This insulinotropic effect of whey is due to the direct effect of amino acids on β-cells to secrete insulin and stimulate incretin, as it inhibits the action of DPP-4, so reducing PBG [11]. Also, whey protein helps to improve inflammation and oxidative stress, which play a critical role in developing diabetes complications [12].

The insulin receptors contain a tyrosine kinase enzyme. Binding of insulin to the α subunit of the receptor activates the β subunit, which leads to autophosphorylation and activation of tyrosine kinase [13]. Insulin promotes the uptake of blood glucose into the skeletal muscle after binding with insulin receptor tyrosine kinase (IRTK). Skeletal muscle is responsible for 85% of insulin-enhanced uptake of glucose from the blood and it is the principal tissue responsible for reducing blood glucose level [14].

The current study was conducted to highlight the effect of different concentrations of whey protein (10%, 20%, and 40%) as an antidiabetic agent compared to vildagliptin against high-fat diet and streptozotocin (HFD/STZ) induced T2DM in rats and to clarify the possible molecular mechanisms underlying whey protein action.

Material and Method

Animal model

Sixty male Wistar rats weighing 90–110 g at approximately five weeks of age were obtained from the National Research Center, Giza, Egypt. The rats were weighed and housed in aluminum cages for two weeks under identical environmental conditions for adaptation and allowed free access to normal chow diet and water.

Experimental design

The study was performed under the guidelines for the care and use of laboratory animals and approved by the Research Ethics Committee of Faculty of Pharmacy, Tanta University, Egypt. After the acclimatization period, rats were weighed and randomly divided into two major groups: normal control group (n=10), and diabetic (n=50). The untreated normal control group was maintained on the control diet and water ad libitum and received a single i.p. dose of the formulation containing the vehicle (0.1 M sodium citrate buffer 0.25 mL/kg). The diabetic rats received HFD for two weeks and then 35 mg/kg STZ injection to develop T2DM model (HFD/STZ). The STZ injection is a single i.p. dose of STZ (Sigma Aldrich®, USA) dissolved in 0.1 M sodium citrate buffer, pH 4.5. That model aimed to develop T2DM pathology progression, and insulin resistance or hypoinsulinemia, in a condensed timeline [15]. The composition of the control diet and HFD are shown in Table 1.

After three days, STZ induced β-cell-toxicity and induces experimental diabetes. STZ is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release so, the rats were maintained on 5% glucose solution via their drinkers for the 24 hour following STZ administration to prevent hypoglycemia [16].

Blood glucose was tested using a blood glucose meter (Accu-Chek Performa; Roche Diagnostics®, USA). All rats with blood glucose concentrations greater than 16.7 mmol/l were considered to be diabetic and were selected for further research according to the American Diabetes Association [17]. Diabetic rats were further randomly divided into five subgroups (n=10); Group 1: untreated diabetic group received 1 mL of the formulation containing the vehicle. Group 2: vildagliptin (Novartis International AG®, Switzerland) treated group received 1 mL of the formulation vildagliptin (10 mg/kg) once a day via oral gavage for four consecutive weeks [18].

Groups 3, 4 and 5: whey protein 10%, 20% and 40% treated groups respectively received 1mL of the formulation containing whey protein (1 mL/100 g) once a day via oral gavage for four consecutive weeks. Whey protein was obtained

Table 1: The composition of the control diet and high fat diet (HFD)

Diet components	Control diet	HFD
Energy per day (Kcal/g)	3	4
Calorie percent:		
protein	22	20 (casein)
Fat	12	45 (lard)
Carbohydrate	66	35 (corn starch, maltodextrin, and sucrose)

from Optimum Company®, USA and was dissolved in distilled water to prepare the different concentrations (10%, 20%, and 40%).

Specimen collection

At the end of the experiment, rats were weighed and the blood was withdrawn from the eye veins after overnight food deprivation for determination of FBG. The remaining blood was immediately centrifuged for 15 minute at 3000 rpm and serum was stored at -80°C for the determination of fasting insulin. The fasted rats were allowed to eat and the PBG was determined after two hour. Rats were then sacrificed and their pancreas, small intestines, and skeletal muscles were dissected. The fresh pancreas was washed twice in ice-cold saline dried on clean paper towels and kept in formalin solution for histopathological examination. The small intestine was kept frozen at -80°C till the determination of the concentration of GIP and GLP-1. Skeletal muscles were kept frozen at -80°C till the measurement of IRTK concentration.

Determination of blood glucose concentration

Accu-Chek advantage capillary glucose meter (Roche Diagnostics®, Germany) was used for the immediate determination of FBG and PBG in whole blood using the noble metal electrode strip.

Determination of serum insulin level

Serum insulin concentration was measured by rat insulin ELISA kit, obtained from MLbio Biotechnology®, China. The concentration of insulin was determined according to manufacturer procedure and expressed as μIU/mL. Insulin resistance was determined by homeostasis model assessment-insulin resistance (HOMA-IR) index described by Matthews et al [19], which was calculated as follows:

$$\text{HOMA-IR} = \frac{\text{FBG (mg/dL) X fasting insulin (μU/mL)}}{405}$$

Determination of glucose dependent insulinotropic polypeptide (GIP) in the small intestine

It was determined in small intestine tissue by rat GIP ELISA kit obtained from MLbio Biotechnology®, China. The concentration of GIP was determined according to the manufacturer procedure and was expressed as pg\g tissue.

Determination of glucagon like peptide-1 (GLP-1) in the small intestine

It was determined in the rat's small intestine tissue by rat GLP-1 ELISA kit obtained from MLbio Biotechnology®, China. The concentration of GLP-1 was determined according to the manufacturer procedure and was expressed as pg\g tissue.

Determination of insulin receptor tyrosine kinase (IRTK) in skeletal muscles

Rat IRTK ELISA kit obtained from MLbio Biotechnology®, China was used to measure IRTK in rat skeletal muscles. The concentration of IRTK was determined according to the manufacturer procedure and was expressed as pg\g tissue.

Histopathological examination

Transverse pancreas sections were carefully embedded in molten paraffin and kept

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under freezing plates to allow the paraffin to solidify. Cross sections (5 µm thick) of the fixed tissues were cut. These sections were stained with hematoxylin and eosin (H&E) stain for general histopathological examination. Images were viewed and recorded using Olympus microscope equipped with spot digital camera using computer program MATLAB software by abroad certified pathologist in Pathology Department, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt. The investigator performing the histological evaluation was blind to biochemical results and treatment allocation.

Statistical analysis

Analysis of data was performed with the statistical package for social science (SPSS) software version 22 [20]. Data are presented as % change and mean ± SEM. Statistical comparison among groups was performed using one-way analysis of variance (ANOVA) using Fisher's least-significant differences (LSD) method for comparison between two groups. Statistical significance was set at $p< 0.05$.

Results

Effect of whey protein and vildagliptin on blood glucose, insulin level, and HOMA-IR

Diabetic control rats showed a significant increase ($p<0.05$) in the level of FBG and PBG (189.3±1.78 mg/dL and 335.2±17.9 mg/dL, respectively) versus the normal control group (86±1.86 mg/dL and 120±3 mg/dL, respectively). Treatment with vildagliptin significantly decreased ($p<0.05$) FBG and PBG level (92.8±1.5 mg/dL and 128.1±21 mg/dL, respectively) versus the diabetic control group. Treatment with 10%, 20% and 40% whey protein significantly decreased ($p<0.05$) FBG level (158.8±1.6 mg/dL, 126.4±1.9 mg/dL, and 79.6±1.27mg/dL, respectively) compared to the diabetic control group. Besides, treatment with 10%, 20% and 40% whey protein significantly decreased ($p<0.05$) PBG level (184.7±1.82 mg/dL, 151.6±2.71 mg/dL, and 122.4±2.43 mg/dL, respectively) compared to the diabetic control group (Fig. 1A & B).

Injection with STZ caused a significant decrease ($p<0.05$) in fasting blood insulin level in the diabetic group (7.39±0.02µIU/mL) versus the normal control group (8.81±0.06µIU/mL). Treatment with vildagliptin significantly increased ($p<0.05$) the level of fasting insulin (8.83±0.05µIU/mL) versus the diabetic control group. Treatment with 10%, 20% and 40% whey protein significantly increased ($p<0.05$) the level of fasting insulin (7.83±0.05µIU/mL, 8.36±0.03µIU/mL, and 9.37±0.03µIU/mL, respectively) versus the diabetic control group (Fig. 1C).

HOMA-IR values were calculated for the estimation of insulin resistance in the different groups. Injection with STZ caused a significant increase ($p<0.05$) in the HOMA-IR value of the diabetic group (3.46±0.1)versus the normal control group (1.87±0.03). Treatment with vildagliptin significantly decreased ($p<0.05$) HOMA-IR value (2.01±0.1) compared to the diabetic control group. Treatment with 10%, 20% and 40% whey protein significantly decreased ($p<0.05$) HOMA-IR value (3.071±0.03, 2.608±0.04, and 1.842±0.03, respectively) compared to the diabetic control group (Fig. 1D). Interestingly, 40% whey protein group showed a reduction in the blood glucose and HOMA-IR value to a level comparable to that of the normal control group (Fig. 1).

Effect of whey protein and vildagliptin on intestinal GIP and GLP-1 concentrations

The diabetic control group showed a significant decrease ($p<0.05$) in GIP concentration (3.64±0.12 ng/g) versus the normal control group (6.16±0.12 ng/g). Treatment with vildagliptin showed a significant increase ($p<0.05$) in GIP concentration (18.8±0.13 ng/g) versus the diabetic control group. Treatment with 10%, 20%, and 40% whey protein significantly increased ($p<0.05$) GIP concentration (7.71±0.18 ng/g, 12.12±0.32 ng/g, and 14.82±0.24 ng/g, respectively) versus the diabetic control group (Fig. 2A).

The diabetic control group showed a significant decrease ($p<0.05$) in GLP-1

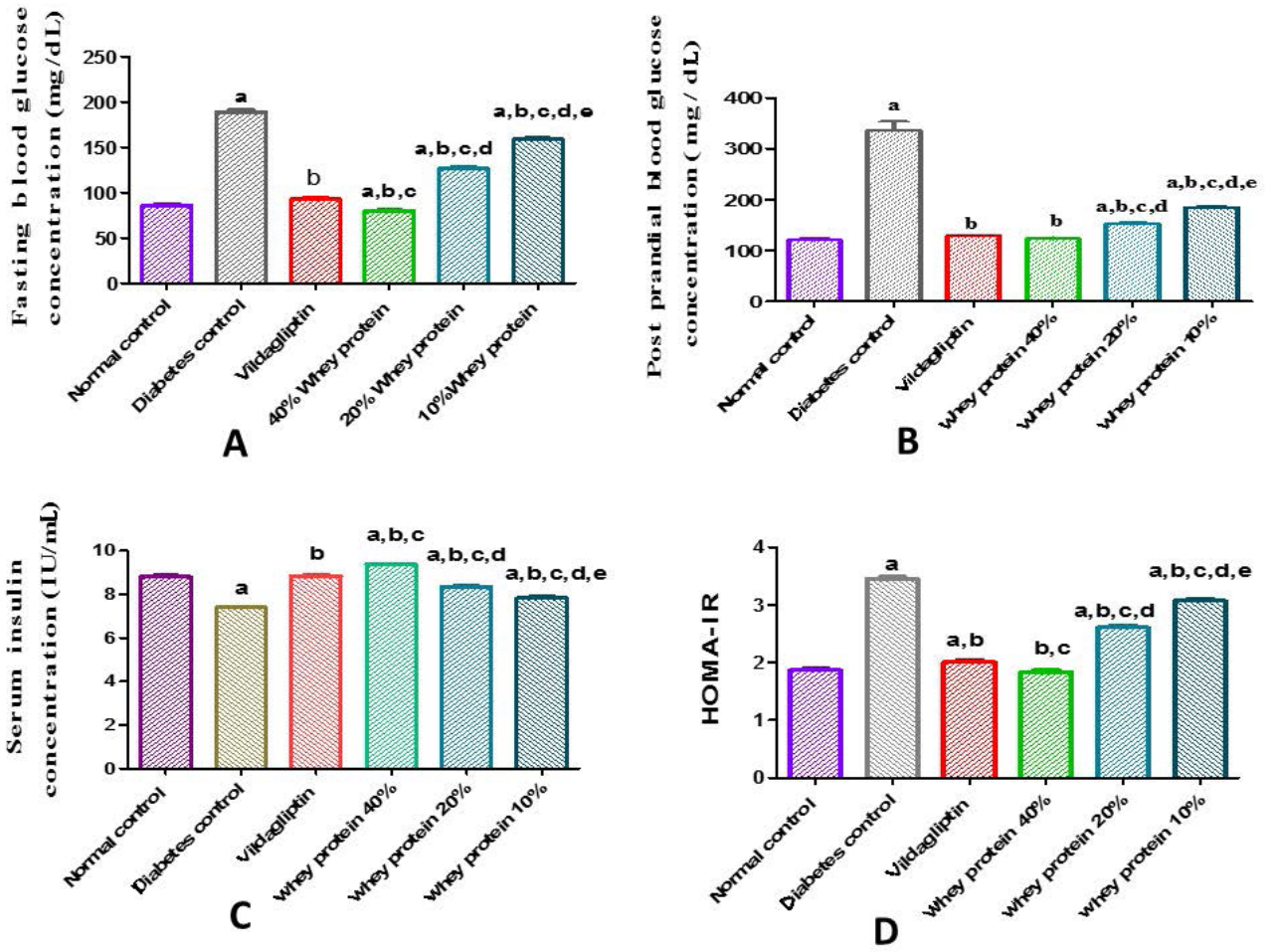

Figure 1: (A) Fasting blood glucose level in rat groups. (B) Postprandial blood glucose level in rat groups. (C) Fasting insulin level in the serum of rat groups. (D) HOMA-IR in rats groups. Data are presented as mean ± SEM (n= 10/group), significance was set at $p<0.05$; a: Significant versus normal control group, b: Significant versus diabetic control group, c: Significant versus vildagliptin group, d: Significant versus 40% whey protein group, e: Significant versus 20% whey protein group.

concentration (9.22±0.37 pg/g) versus the normal control group (13.41±0.22 pg/g). Treatment with vildagliptin showed a significant increase ($p<0.05$) in GLP-1 concentration (33.38±0.64 pg/g) versus the diabetic control group. Treatment with 10%, 20% and 40% whey protein significantly increased ($p<0.05$)GLP-1 concentration (16.95±0.6 pg/g, 24.24±0.54 pg/g, and 28.55±0.29 pg/g, respectively) versus the diabetic control group (Fig. 2B).

Effect of whey protein and vildagliptin on IRTK in skeletal muscles

Injection with STZ caused a significant decrease ($p<0.05$) in IRTK concentration in skeletal muscles isolated from the diabetic control group (1340±92.74 pg/g) versus the normal control group (2583±85 pg/g). Treatment with vildagliptin showed a non-significant increase in IRTK concentration (1488±101 pg/g) versus the diabetic control group. On the other hand, treatment with 10%, 20% and 40% whey protein significantly ($p<0.05$) increased IRTK concentration (2130±181.2 pg/g, 3168±90.45 pg/g, and 4273±82.28 pg/g, respectively) versus the diabetic control group (Fig. 2C).

Effect of whey protein and vildagliptin on the histopathology of the pancreas

By using MATLAB software the number of β-cells per islet can be determined. Injection with STZ caused a significant decrease ($p<0.05$) in the number of β-cells (27.33±2.23) versus the normal control rats (172.2± 9.86) (Fig. 2D). The sections

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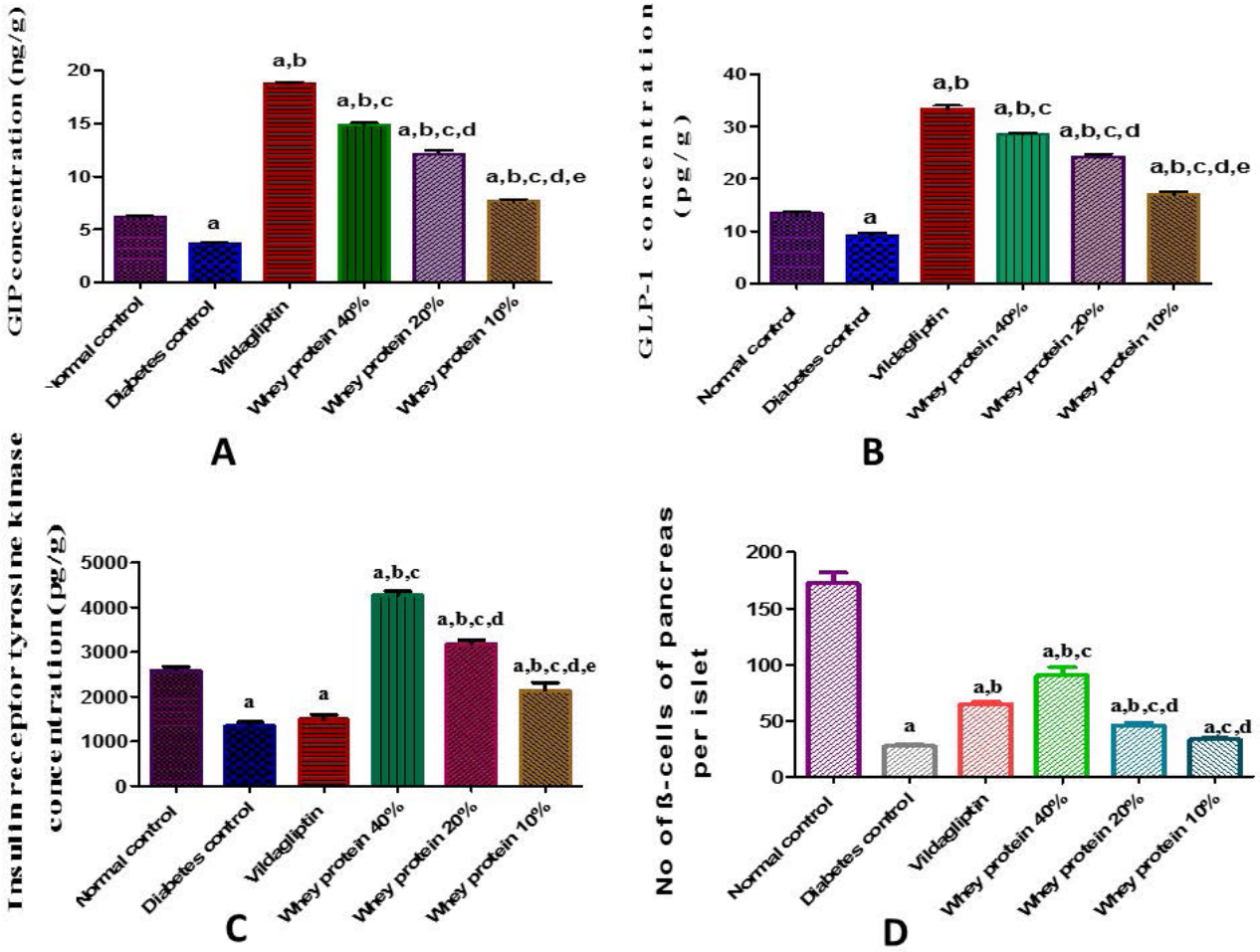

Figure 2: (A) Concentration of intestinal GIP in rats groups. (B) Concentration of Intestinal GLP-1 in rats groups. (C) Concentration of insulin receptor tyrosine kinase in skeletal muscles of rats groups. (D) Number of β-cells of the pancreases per islet in rats groups. Data are presented as mean ± SEM (n= 10/group), significance was set at $p<0.05$; a: Significant versus normal control group, b: Significant versus diabetic control group, c: Significant versus vildagliptin group, d: Significant versus 40% whey protein group, e: Significant versus 20% whey protein group.

from the pancreas of the normal control group showed normal islets of Langerhans containing normal population of α and β-cells, normal blood capillaries embedded in exocrine portion with normal intercalated duct (Fig. 3A). The sections from the pancreas of the diabetes control group showed signs of inflammation indicated by the deposition of pale eosinophilic amyloid substance in islets of Langerhans with degeneration and necrosis of β-cells, normal exocrine portion of the pancreas, and normal intercalated duct lined by simple cuboidal epithelium (Fig. 3B).

Treatment with vildagliptin or 40% whey protein showed a significant increase ($p<0.05$) in the number of β-cells as shown by the large proportion and number of islet cells and restoring the histological architecture of islets of Langerhans (Fig. 3C and D). Treatment with 20% whey protein showed significant increase ($p<0.05$) in the number of β-cells and moderate restoring of the histological architecture of islets of Langerhans with mild deposition amyloid substance (Fig. 2D and Fig. 3E).

On the other hand, the section from the pancreas of 10% whey protein treated group showed the presence of amyloid substance with mild restoration of β-cells of islets of Langerhans which is non-significant when compared to the diabetic group (Fig. 2D and Fig. 3F).

Discussion

The prevalence of diabetes is increasing around the world, affecting approximately 424.9×10^6 people in 2017 and expected to increase

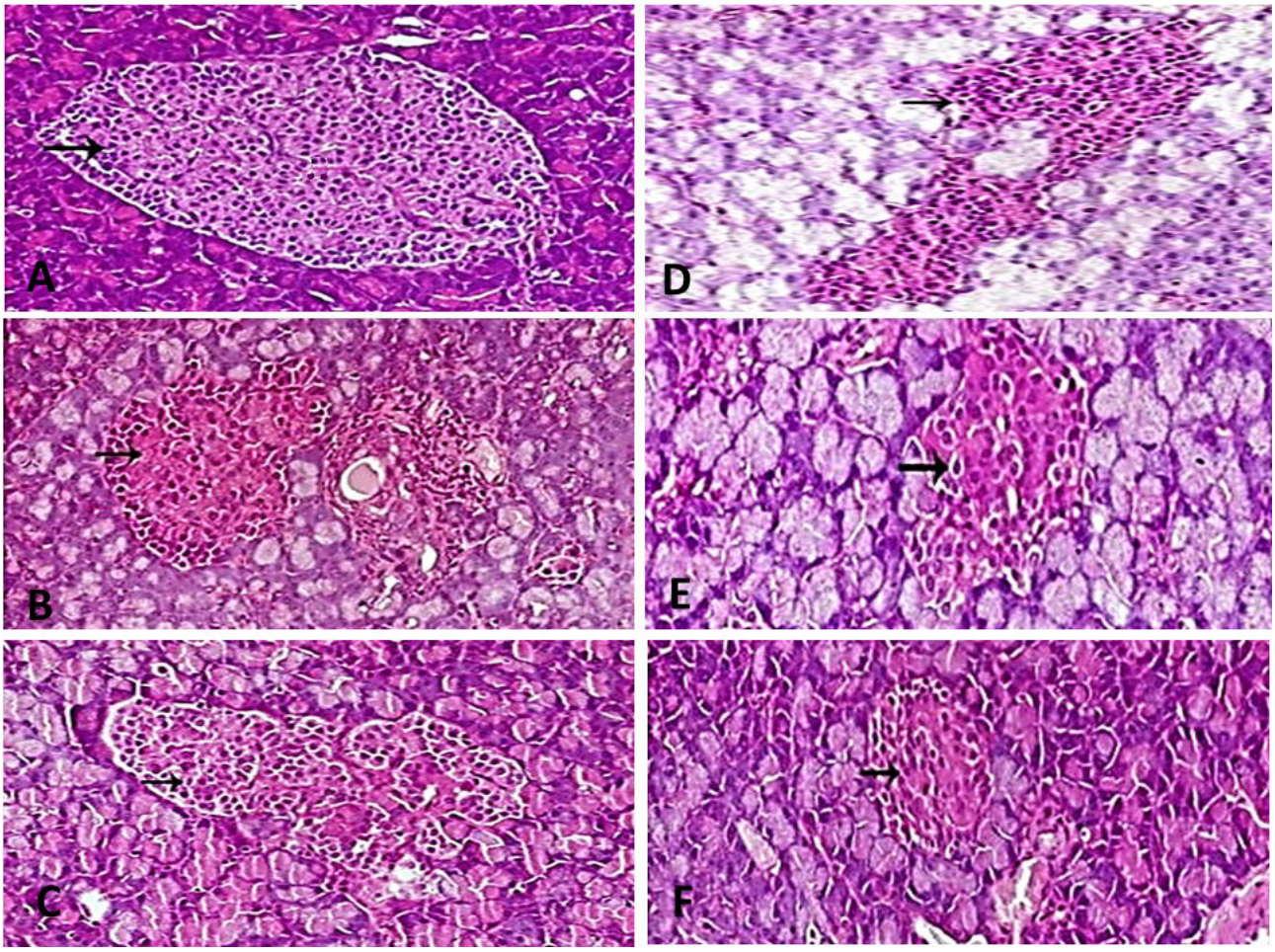

Figure 3: Sections from the pancreas (H&E, 400x) of (A) normal control group showing normal islets of Langerhans containing a population of α and β-cells (arrow) and were all present in their normal proportions. (B) diabetic control group showing deposition of pale eosinophilic amyloid substance in islets of Langerhans (arrow) with degeneration and necrosis of β-cells. The dimension and the number of cells in each islet of Langerhans were also reduced. (C) vildagliptin treated group showing an increase in the number of β-cells (arrow) and restoring the histological architecture of islets of Langerhans, The number and size of islet of Langerhans were increased as compared to the diabetic control group. The number of cells in each islet was also increased. (D) 40% whey protein treated group showing an increase in the number of β-cells (arrow) and restoring the histological architecture of islets of Langerhans. The islets are present with a large proportion and number of islet cells. (E) 20% whey protein treated group showing a mild increase in the number of β-cells (arrow) and a mild restoring the histological architecture of islets of Langerhans with mild deposition amyloid substance. (F) 10% whey protein treated group showing a small increase in the number of β-cells (arrow) with necrosis of β-cells and a mild deposition amyloid substance.

to 628.6 x10^6 in 2045 [21]. Nowadays, alternative and complementary medicine provide us with many natural products, including whey protein which approved its therapeutic value in reducing blood glucose levels in diabetes [12]. This study was conducted to compare the therapeutic effects of different concentrations of whey protein (10–20%, and 40%) with vildagliptin (DPP4 inhibitor) in T2DM rat model and to clarify the molecular mechanisms underlying whey protein action.

Experimental T2DM was induced in the present study in male Wistar rats by (HFD/STZ) and the diabetes control group showed a significant elevation of both FBG and PBG versus normal controls. Diabetes induction was also evidenced by the histopathological changes of the pancreas which showed deposition of pale eosinophilic amyloid substance and vacuolizations in islets of Langerhans with degeneration and necrosis of β-cells. The number of β-cells per islets in the diabetes control group was significantly lower than that of the normal control group.

Our findings demonstrate that treatment with vildagliptin or whey protein 10%-20%-40%)

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reversed the effect of STZ significantly on blood glucose levels in diabetic control rats. Our study showed that treatment with whey protein significantly decreased FBG and PBG in a concentration-dependent manner and 40% whey protein decreased the level of blood glucose to a level comparable or lower than that of vildagliptin treated group. Our results were in agreement with King et al [11] who reported that a small dose of whey protein before meals improves PBG in men with T2DM.

These results were matched with our observed histopathological findings as the section of the pancreas of vildagliptin or 40% whey protein treated group showed a significant increase in the number of β-cells as shown by the large proportion and number of islet cells and restoring the histological architecture of islets of Langerhans. Treatment with 20% showed an increase in the number of β-cells and moderate restoring of the histological architecture of islets of Langerhans with mild deposition amyloid substance. While the pancreas of 10% whey protein group showed the presence of amyloid substance with mild restoration of β-cells of islets of Langerhans which is non-significant when compared to the diabetic group.

In our study, we determined the number of β-cells per islets by MATLAB software. Herein, treatment with vildagliptin or whey protein (20%-40%) showed a significant increase in the number of β-cells versus the diabetic control group. Our study showed that treatment with whey protein (10%-20%-40%) increased the number of β-cells in a concentration-dependent manner and 40% whey protein increased the number of β-cells to a level significantly higher than that of vildagliptin treated group. Our results were in agreement with Argun-Kurum et al [22] who showed that vildagliptin promotes proliferation of islet cell and rearranges the morphology of islet in diabetic rats.

As shown by our results, injection with STZ caused a significant decrease in fasting blood insulin levels in the diabetic group versus the normal control group. Treatment with vildagliptin or whey protein (10%-20%-40%) significantly reversed the effect of STZ on insulin secretion. Interestingly our results showed that treatment with whey protein significantly increased insulin secretion in a concentration-dependent manner and 40% whey protein increased the level of blood insulin to a level significantly higher than that of the vildagliptin treated group. This goes with the results reported by Wildova et al [23] who found that oral administration of whey protein increases the serum C-peptide in diabetic patients.

For the estimation of insulin resistance, we calculated HOMA-IR values in the different groups. Our results showed that injection with STZ caused a significant increase in the HOMA-IR value of the diabetic group versus the normal control group while treatment with vildagliptin or whey protein (10%-20%-40%) significantly decreased HOMA-IR value compared to the diabetic control group. Our results were in line with Tong et al [24] who reported that the supplementation of 15% whey protein significantly decreases HOMA-IR in non-obese IR rats.

The incretin (GIP and GLP-1) increase insulin secretion after oral ingestion of carbohydrate [3]. In the present study, the diabetic control group showed a significant decrease in GIP and GLP-1 concentration versus the normal control group. Furthermore, treatment with vildagliptin or whey protein (10%-20%-40%) showed a significant increase in GIP and GLP-1 concentration versus the diabetic control group. Moreover, treatment with whey protein significantly increased GIP and GLP-1 levels in a concentration-dependent manner. In support of our finding, Giezenaar et al [25] demonstrated that whey protein causes an increase in concentrations of insulin, GIP and GLP-1 in older men and women.

One of the mechanisms of insulin to decrease blood glucose is to induce glucose uptake by the skeletal muscles via activation of IRTK [26]. In the current study, injection with STZ caused a significant decrease in IRTK concentration in skeletal muscles isolated from the diabetic control group versus the normal control group. Our study showed for the first time that, treatment with whey protein (10%-20%-40%) showed a significant increase in IRTK concentration versus the diabetic control group and vildagliptin treated group. Besides, the increment in

skeletal muscle content of IRTK due to whey protein treatment is concentration-dependent.

Conclusion

The insulinotropic and anti-hyperglycemic effect of whey protein in T2DM rats is concentration-dependent which could be mediated by increasing each of intestinal concentration of incretin hormones (GIP and GLP-1), skeletal muscle content of IRTK, and number of β-cells of the pancreas. Our results showed that 40% whey protein was comparable to vildagliptin in lowering blood glucose level and HOMA-IR value and increasing insulin secretion. However, 40% whey protein was superior to vildagliptin in increasing the level of muscle IRTK thereby increasing muscle sensitivity to insulin. Being a natural product, 40% whey protein could be used safely as a substitute for synthetic drugs for the treatment of T2DM patients. Future clinical studies are recommended to investigate the beneficial therapeutic efficacy of 40% whey protein over vildagliptin.

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Conflict of Interest

The authors declare no conflict of interest.

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