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A novel long acting DPP-IV inhibitor PKF-275-055 stimulates β-cell proliferation resulting in improved glucose homeostasis in diabetic rats

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ABSTRACT

The enzyme dipeptidyl peptidase-IV (DPP-4) inactivates the incretin hormone glucagon-like peptide-1 (GLP-1). GLP-1 has therapeutic effects in patients with type 2 diabetes, but its potential is limited by a short half-life, DPP-4 inhibition is a promising approach to diabetes treatment. This study examined chronic (once-a-day dosing for 8 weeks) effects of the DPP-4 inhibitor PKF-275-055 (1, 3, and 10 mg/kg) on β-cell regeneration and plasma DPP-IV activity, intact GLP-1, glucose, and insulin after an oral glucose load in neonatal wistar rats injected with streptozotocin (STZ) (n2-STZ model), a recognized model of type 2 diabetes. In streptozotocin induced diabetic rats, PKF-275-055 (3, and 10 mg/kg) significantly reduced glucose excursion during the oral glucose tolerance test conducted 2 h and 10 h after administration, with increases in plasma insulin and active glucagon-like peptide-1 (GLP-1) levels and significantly inhibited (> 50% inhibition) plasma DPP-IV activity during both the 1st and 2nd OGTT in diabetic rats. In contrast, PKF-275-055 (1–10 mg/kg) did not cause hypoglycemia in fasted normal rats. Furthermore, PKF-275-055 significantly inhibited advance glycation end product (HbA1c), HOMA-Index, gastric emptying and small intestinal transit rates, with significance at doses of 1 mg/kg or higher. Immunological staining showed PKF-275-055 stimulates β-cell regeneration and reduces pancreatic cell apoptosis in diabetic treated rats. The present preclinical studies indicate that PKF-275-055 is a novel selective DPP-IV inhibitor with long-acting antidiabetic effect that might be a potential agent for type 2 diabetes

1. Introduction

Dipeptidyl-peptidase 4 (DPP4) belongs to the prolyl oligopeptidase family of serine proteases. It removes the N-terminal dipeptide from peptides that have proline or alanine in the second position. Although DPP4 is an extracellular membrane protein, it is also found in human plasma as a soluble form that lacks the transmembrane region. DPP4 modulates the biological activity of several peptide hormones, chemokines, and neuropeptides by cleaving the molecule after the proline or alanine residue [1]. Glucagon-like peptide-1 (GLP-1) is an incretin hormone released from the gut during meals that serves as an enhancer of glucose stimulated insulin secretion from pancreatic β-cells. A chronic infusion of GLP-1 for treatment of type 2 diabetes resulted in improved blood glucose and hemoglobin A1c (HbA1c) [2]. However, GLP-1 is rapidly degraded in plasma by DPP4. Since the inhibition of DPP4 increases the levels of endogenous circulating GLP-1, DPP4 could be a new therapeutic target for the treatment of type 2 diabetes [3]. DPP4 inhibitors improved glycemic control, insulin secretion, and β-cell function in rodents [4,5]; additionally, vildagliptin, or other DPP-IV inhibitors have been shown to increase proliferation, neogenesis, β-cell mass, insulin biosynthesis, and insulin content and decrease apoptosis in different animal models [6–9]. In patients with type 2 diabetes, chronic treatment with DPP4 inhibitors decreased postprandial glucose excursion, fasting plasma glucose, and HbA1c. It was well-tolerated with neutral weight effects, a low incidence of hypoglycemia, and gastrointestinal adverse events [2,10]. Recent studies have suggested that exogenous GLP-1 or GLP-1 derivatives cause a delay in gastric emptying both in healthy volunteers and type 2 diabetes patients [11–13]. On the other hand, no delay in gastric emptying occurred when the endogenous GLP-1 level increased following administration of DPP-IV inhibitors [14]. One possible explanation for this discrepancy is that there may be a difference in the plasma GLP-1 levels that induce the incretin and gastrointestinal effects. Therefore, it is important to investigate the effect of DPP-IV inhibitors on gastrointestinal functions and plasma GLP-1 levels.

Short half life; inefficient inhibition of gastrointestinal functions, and diabetic complications with the administration of vildagliptin has always been a matter of concern [15,16]. Hence it was imperative to search for new long acting DPP-4 analogues. The discovery of PKF-275-055, analogue of vildagliptin was in persuasion of the unmet need. The purpose of the present study

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is to characterize the pharmacological profile of vildagliptin analogue PKF-275-055 which was synthesized as a selective, long-acting inhibitor of dipeptidyl peptidase 4 that was discovered by Novartis switzerland. This characterization was conducted with regard to the following points: (1) assessment of the effects on blood glucose, plasma insulin, DPP-IV inhibition and GLP-1 levels after oral glucose loading in rats (2) evaluation of HbA1c level, insulin resistance and β-cell function, (3) investigation of effects on gastric emptying and small intestinal transit rate. (4) investigate β-cell proliferation, apoptosis, and neogenesis.

2. Materials and methods

2.1. Materials

PKF-275-055 (Novartis Switzerland), vildagliptin (LAF237; 1-[[(3-hydroxy-1-adamantyl) amino] acetyl]-2 cyano-(S)-pyrrolidine), were synthesized by Novartis Switzerland. These compounds were dissolved or suspended in 0.5% carboxymethylcellulose in 0.2% Tween 80, and then orally administered. Insulin ELISA kit (SPI BIO, france, Catalog #A05105), GLP-1 ELISA kit (Linco Research, Japan, catalog #YK050, lot 091130), DPP-IV ELISA kit (R&D Systems, USA, Catalog # DC260), HbA1c assay kit (Biosystem, Spain, catalog # COD 11044), Apo-BrdU-IHC™ *In Situ* DNA Fragmentation Assay Kit (Biovison, USA, Catalog #K403-50), chromogen (sigma chemicals, USA), Hematoxylin and eosin (sigma chemicals, USA), streptavidin peroxidase (Genetex, USA), Tween 80 (Merck, India), phenol red (Applichem, Germany), methylcellulose (sigma chemicals, USA), trichloroacetic acid (Merck, India), tris buffer (Applichem, Germany), and 3,3′-diaminobenzidine (sigma chemicals, USA).

2.2. Animals

Healthy albino rats of Wistar strain were kept for breeding. To induce NIDDM, STZ (sigma chemicals, USA) (90 mg/kg) was administered *i.p.* to a group of 2 days old pups. Another group of pups received only saline. The pups were weaned for 21 days, and 6 weeks after the injection of STZ, the animals were checked for fasting glucose level (FPG) $\geq$160 mg/dl were considered as diabetic. Pups that receive saline were considered as control animals, after which they were grouped so that the blood glucose levels were uniform among the groups. All rats were housed under conventional conditions with controlled temperature, humidity and light (12 h light–dark cycle), and were provided with a standard commercial diet and water (*ad libitum*). All experimental procedures were conducted according to the Institutional Animal Ethical Committee (protocol no. DIPSAR/IAEC/2009/25) and CPCSEA guidelines.

2.3. Eight week chronic daily dosing study

After 6 weeks, the animals were assigned to receive vehicle or PKF-275-055 or vildagliptin at the dose level of 1, 3, and 10 mg/kg once daily for 8 weeks to evaluate dose dependant activity [17]. On the morning after final administration, blood samples were collected under fasting conditions and body weight was measured; and the pancreas was isolated and was immersed and fixed in phosphate-buffered 10% formalin solution to prepare a paraffin section.

2.4. Immunocytochemistry

Whole pancreas from rats was removed under anesthesia and fixed in 10% buffered formalin for 24 h. Tissues were dehydrated in graded series of alcohol, embedded in paraffin, sectioned at 5 μm thickness and used for immunostaining. The tissue sections were stained with hematoxylin and eosin while the remaining serial sections were used for immunostaining. Serial sections of the rat pancreas were immunostained by strepavidin-biotin peroxidase method using pre-diluted polyclonal antibodies. All sections were de-paraffinized in xylene bath to remove the excess wax. The slides were placed in two changes of absolute alcohol for 3 min each. The same procedure was repeated with 90% alcohol. The slides were placed in blocking reagent in order to block the endogenous peroxidase activity for 5 min, which was pre-diluted with 5 volumes of 100% ethanol. The slides were placed in two changes of 70 per cent alcohol for three min each. The excess alcohol around the sections was removed and the slides were quickly immersed in Tris buffer, pH 7.6 for 5 min. Two drops of tissue conditioner was added and the sections were incubated for 5 min and then rinsed in buffer solution. Pre-diluted primary polyclonal anti-guinea pig antibody to insulin (1:1000) [Genetex, USA] raised against human insulin was added to the sections and incubated for 1 h. The secondary antibody for insulin was anti-rabbit polyclonal antibodies. After incubation for half an hour, the sections were rinsed with tris buffer, peroxidise solution was added, incubated for 30 min and later rinsed with the buffer. AEC (3-amino, 9-ethyl carbazole) chromogen substrate was added to the sections and was incubated for 15 min and rinsed with distilled water. The sections were counter-stained with Harris' hematoxylin for 45 s to facilitate nuclear identification [18].

2.5. DNA fragmentation assay

For detection and localization of apoptosis in pancreas, we used the technique of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Apo-BrdU-IHC™ *In Situ* DNA Fragmentation Assay Kit, Biovison, USA). Briefly, sections were deparaffinized, hydrated, and digested with proteinase K (20 μg/ml), and then added biotinylated dUTP to the 3′ end of DNA fragments by incubating sections in 0.05 mol/l Tris–HCl buffer (pH 7.6) with 0.03 U/μl TdT and 0.04 nmol/μl biotin-11-dUTP at 37 °C for 1 h. The sections were rinsed in PBS. Endogenous peroxidase was blocked with 0.3% H_2O_2 in distilled H_2O. The sections were rinsed with PBS and covered with 2% blocking solution in 0.1 mol/l sodium maleate to reduce background staining. The sections were then incubated with avidin-peroxidase complexes in PBS (1:50) for 30 min and rinsed with PBS (3 × 5 min). Peroxidase activity was visualized with 3,3′-diaminobenzidine until the brown product was clearly visible. The sections were then counterstained with methyl green. The positive apoptotic cells were the cells with brown nucleus [19].

2.6. Blood glucose levels during the oral glucose tolerance test (OGTT) in diabetic rats

Blood samples were collected from normal and diabetic rats fasted overnight for the measurement of blood glucose levels and to which either the vehicle or the test compound had been orally administered. After 30 min, blood glucose levels were measured again, after which glucose solution (2 g/kg) was orally administered (1st OGTT). At 0.5, 1, 2 and 4 h after glucose loading, blood glucose levels were measured. At 8 h after the first glucose loading (8.5 h after drug administration), blood glucose levels were measured, and then glucose solution was orally administered (2nd OGTT). The blood glucose levels were again measured at 0.5, 1, 2 and 4 h after the second glucose loading [20].

2.7. Plasma insulin, GLP-1 and DPP-IV levels during the OGTT in diabetic rats

Normal and diabetic rats were fasted overnight, and blood samples (0.2 ml at each interval) (basal value) were collected to measure plasma insulin, GLP-1 and DPP-IV levels. Either the vehicle or the test Compound was administered orally, and blood samples were collected 30 min later (1st OGTT-pre value). Glucose

solution (2 g/kg) was then orally administered, and blood samples were collected 10 min later (1st OGTT-10 min value). Eight hours after the first glucose loading, blood samples were collected (2nd OGTT-pre value). Glucose solution was then orally administered, and blood samples were collected 10 min later (2nd OGTT-10 min value) [20].

2.8. Blood glucose levels during the oral glucose tolerance test (OGTT) in normal rats

Blood samples were collected from normal rats fasted overnight for the measurement of blood glucose levels and to which either the vehicle or the test compound had been orally administered. After 30 min, blood glucose levels were measured again, after which glucose solution (2 g/kg) was orally administered and the blood glucose levels were measured at 0.5, 1, 2, 4, 6 and 8 h after administration [20].

2.9. Gastrointestinal functions in diabetic rats

Either the vehicle or the test compound was administered to diabetic rat that had been fasted overnight. 30 min later (compound treatment examination), glucose solution (0.2 g/ml glucose, 0.25% methylcellulose, 1 mg/ml phenol red and 10 mg/ml charcoal) was orally administered at a volume of 15 ml/kg. Under ether anesthesia, the stomach was ligated and removed, after which it was transferred to a tube and cryopreserved. The entire length of the small intestine (between the pylorus of the stomach and the end of the ileum) and the distance to the charcoal front were measured. The rats in the control group were given vehicle solution in order to measure the total amount of glucose solution injected into the stomach. At 15 min after administration, the pylorus of the stomach was ligated under ether anesthesia; after which the stomach was immediately removed, and small intestinal transit was checked. To measure the gastric emptying rate, 0.1 mol/L NaOH solution (5 ml) was added to the stomach sample, and they were homogenized. After centrifugation (3000 rpm, 10 min), 20% TCA solution (50 ml) was added to a 500-μl aliquot of the supernatant. The mixture was then stirred and centrifuged (15,000 rpm, 10 min). A 100 μl aliquot of the supernatant was then dispensed into a 96-well assay plate, and 0.5 mol/L NaOH solution (50 μl) was added. After stirring, phenol red concentration in the sample was determined from a phenol red (0–1000 μg/ml) calibration curve. The gastric emptying rate (%) was then calculated using the following equation: [(mean value of the control group) − (the sample value)]/(mean value of the control group). The small intestinal transit rate (%) was calculated using the following equation: (the distance traveled by the charcoal front)/(the entire length of the small intestine) [20].

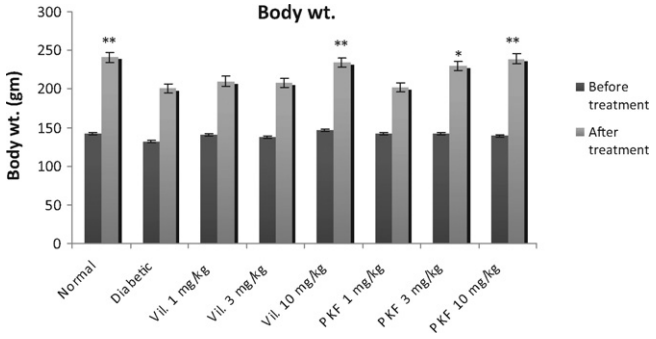

Fig. 1. Body weight before and after treatment with vehicle or different doses of vildagliptin and PKF-275-055. The values are the means ± S.E.M. from eight animals in each group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. diabetic group.

2.10. Glycated hemoglobin (HbA1c) assay

Total HbA1c content, an indicator of irreversible condensation of glucose with the N- terminal residue of the β-chain of hemoglobin A. The HbA1c concentration in blood is directly proportional to the mean concentration of glucose prevailing in the previous 6–8 weeks, equivalent to the lifetime of the erythrocytes [21] and this assay based on the procedures of a commercially available kit (Biosystem, Spain, catalog Number COD 11044).

2.11. Homeostatic model assessment for insulin resistance

The homeostatic model assessment (HOMA) is a method used to quantify insulin resistance and beta-cell function [22]. The approximating equation for insulin resistance, in the early model, used a fasting plasma sample, and was derived by use of the insulin-glucose product, divided by a constant.

$$\text{HOMA} - \text{IR} = \frac{(\text{Glucose} \times \text{Insulin})}{405}; \quad \text{HOMA} - \%\text{B} = \frac{(20 \times \text{Insulin})}{\text{Glucose} - 63}$$

where IR is insulin resistance and %*B* is the β-cell function where glucose is given in mg/dl and Insulin is given in μU/ml (both during fasting).

2.12. Statistical analysis

Values are mean ± S.E.M. Significant differences between treatment groups one-way-analysis of variance (ANOVA) with post-hoc analysis using Dunnet multiple comparison test (sigma plot 11, USA). Values of $p \leq 0.05$ were accepted as significant.

Table 1
Effect of DPP-IV inhibitors on body weight and blood glucose.

	Body weight		Blood glucose	
	Before treatment	After treatment	Before treatment	After treatment
Normal	141.7 ± 3.8	240.8 ± 6.37$^{\#\#}$	84 ± 2.3	79 ± 5.67$^{\#\#\#}$
Diabetic	131.7 ± 4.41	200.8 ± 5.23	164.2 ± 6.54	165 ± 4.83
Vil. 1 mg/kg	140.8 ± 5.68	210 ± 8.56	160.8 ± 4.25***	144.7 ± 2.09$^{\#}$
Vil. 3 mg/kg	137.5 ± 11.01	207.5 ± 8.73	162.7 ± 4.34***	139.7 ± 5.051$^{\#\#}$
Vil. 10 mg/kg	145.8 ± 7.68	234.2 ± 4.36$^{\#\#}$	165 ± 5.16***	117.5 ± 4.03$^{\#\#\#}$
PKF.1 mg/kg	141.7 ± 8.33	202.5 ± 8.03	166.2 ± 4.96***	143.2 ± 4.03$^{\#}$
PKF.3 mg/kg	141.7 ± 8.33	229.2 ± 7.68$^{\#}$	167.7 ± 4.6***	137.8 ± 3.48$^{\#\#}$
PKF.10 mg/kg	139.2 ± 6.5	239.2 ± 5.54$^{\#\#}$	162.2 ± 6.99***	115.5 ± 5.38$^{\#\#\#}$

The values are the means ± S.E.M. from eight animals in each group.
*** $p < 0.001$ vs. normal group
$^{\#}$ $p < 0.05$ vs. diabetic group.
$^{\#\#}$ $p < 0.01$ vs. diabetic group.
$^{\#\#\#}$ $p < 0.001$ vs. diabetic group.

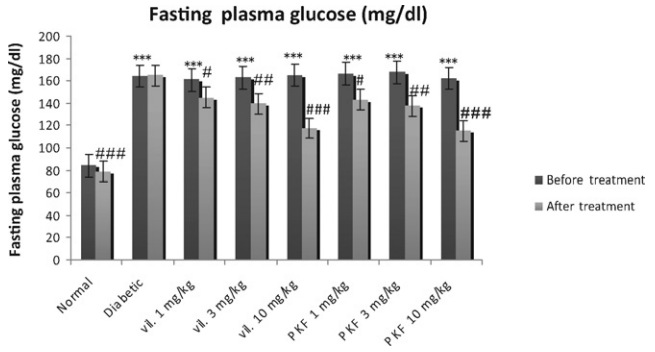

Fig. 2. FPG before and after treatment with vehicle or different doses of vildagliptin and PKF-275-055. The values are the means ± S.E.M. from eight animals in each group.*$p < 0.05$ vs. normal group, #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ vs. diabetic group.

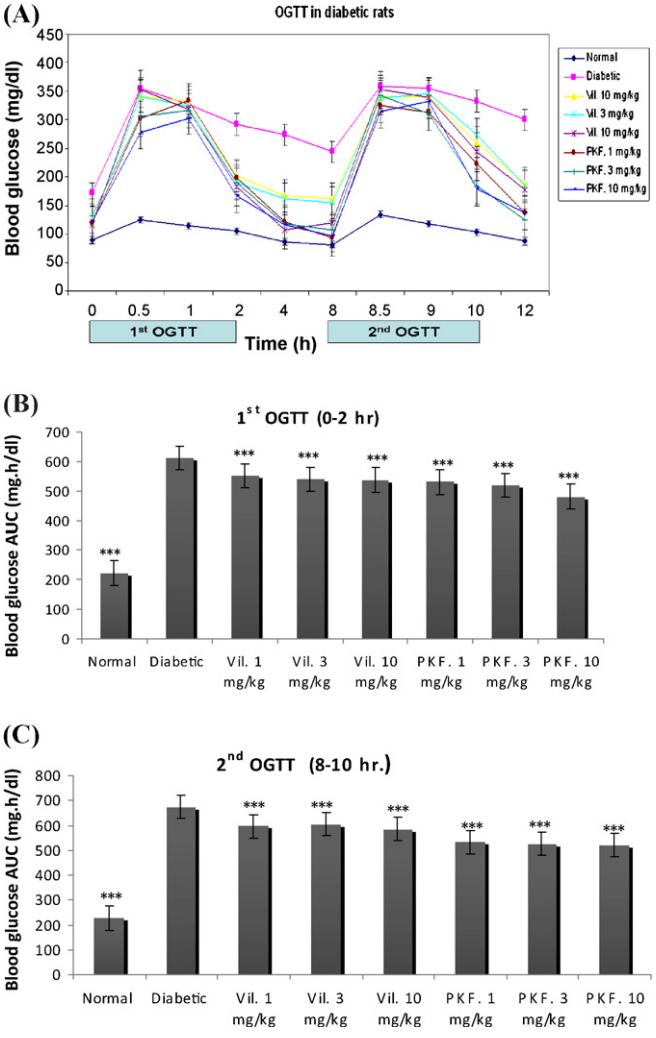

Fig. 3. Effects of vildagliptin and PKF-275-055 on blood glucose levels during the oral glucose tolerance test in streptozotocin induced diabetic rats: (A) time course of changes in blood glucose levels during the oral glucose tolerance test (OGTT) and (B and C) the area under the blood glucose concentration–time curve (AUC) during the OGTT. The values are the means ± S.E.M. from five animals in each group. ***$p < 0.001$ vs. diabetic group.

3. Results

3.1. Effect of DPP-IV inhibitors on body weight and blood glucose

Before the chronic study, there were no significant differences of baseline body weight of the rats (Fig. 1 and Table 1). In the vildagliptin (10 mg/kg) and PKF-275-055 (3 and 10 mg/kg) treated rats showed significant increase in body weight 234.2 ± 4.36 g, 229.2 ± 7.68 g, and 239.2 ± 5.54 g respectively, as compared with diabetic groups (200.8 ± 5.23 g) after 8 weeks study (Fig. 1 and Table 1). Before treatment, there was significantly higher FPG ($p < 0.001$) in all diabetic groups when compared with normal (Fig. 2 and Table 1). After 8 weeks, groups treated with DPP-IV inhibitors showed dose dependent reduction of FPG vs. diabetic group (Fig. 1 and Table 1).

3.2. Effects of DPP-IV inhibitors on blood glucose levels during the OGTT in diabetic rats

The periodic time course of changes observed in blood glucose levels during the oral glucose tolerance test (OGTT) (0–12 h) shows that both vildagliptin (3 and 10 mg/kg) and PKF-275-055 (1, 3, and 10 mg/kg) significantly inhibited the increase in blood glucose level during the 1st OGTT (at 2 h) after the drug administration (Fig. 3A). In contrast, during the 2nd OGTT (10 h), vildagliptin had decrease blood glucose up to 26.21%, whereas PKF-275-055 at the dose of 3 and 10 mg/kg reduced the blood glucose levels up to 44% and 46%; respectively (Fig. 3A). At the same time, area under blood glucose concentration–time curve (AUC) during 1st (0–2 h) and 2nd (8–10 h) OGTT was significantly reduced ($p < 0.001$) in both

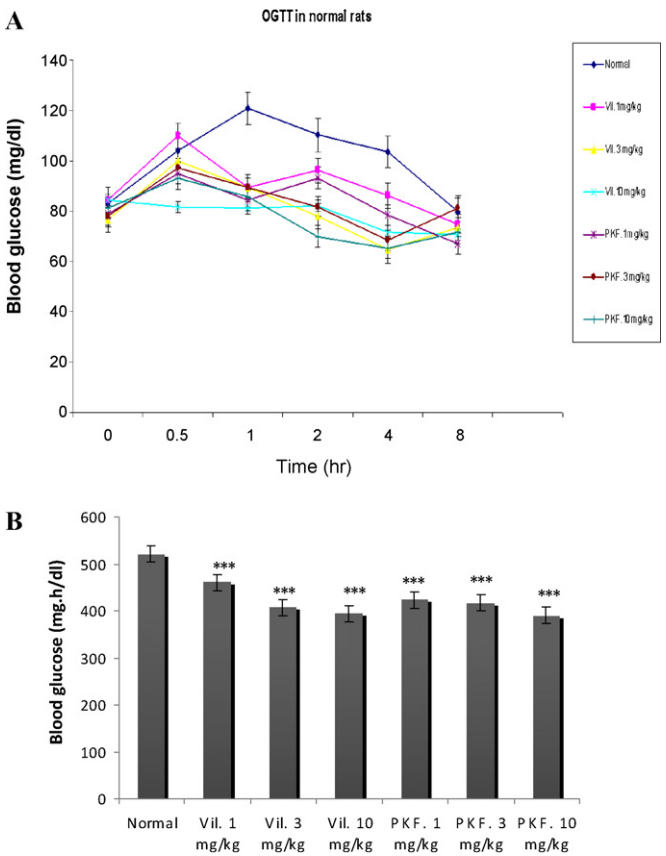

Fig. 4. Effects of vildagliptin and PKF-275-055 on blood glucose levels during the oral glucose tolerance test normal rats: (A) time course of changes in blood glucose levels during the oral glucose tolerance test (OGTT) and (B) the area under the blood glucose concentration–time curve (AUC) during the OGTT. The values are the means ± S.E.M. from five animals in each group. ***$p < 0.001$ vs. normal group.

vildagliptin and PKF-275-055 (Fig. 3B and C) as compared with glucose loaded diabetic rats.

3.3. Effects of DPP-IV inhibitors on blood glucose levels during the OGTT in normal rats

In normal rats, vildagliptin and PKF-275-055 significantly inhibited increases in the blood glucose level during the OGTT conducted at 1 h (Fig. 4A); also both the drugs significantly reduced ($p < 0.001$) the area under the blood glucose concentration–time curve (AUC) during the OGTT vs. vehicle treated group (Fig. 4B).

3.4. Effects of DPP-IV inhibitors on plasma insulin, GLP-1, and DPP-IV levels during the OGTT in diabetic rats

Vildagliptin and PKF-275-055 did not change plasma insulin and GLP-1 levels at pre-OGTT values but both DPP-IV inhibitors at the dose 10 mg/kg significantly increased plasma insulin at 10 min values during the OGTT conducted both 0.5 h (1st) ($p < 0.05$) and 8 h (2nd) ($p < 0.001$) after drug administration (Fig. 5A). In addition, vildagliptin and PKF-275-055 significantly increased GLP-1 concentration in a dose-dependant manner at 10 min values during the OGTT conducted at 0.5 h (1st) ($p < 0.05$, $p < 0.01$, $p < 0.001$) but at 8 h (2nd) vildagliptin (10 mg/kg) and PKF-275-055 (3 and 10 mg/kg) showed significantly increased GLP-1 concentration ($p < 0.001$) after drug administration (Fig. 5B). In addition, vildagliptin and PKF-275-055 significantly inhibited plasma DPP-IV activity at pre-OGTT and 10 min values during the OGTT conducted both 0.5 h (1st) and 8 h (2nd) after drug administration (Fig. 5C).

3.5. Effects of DPP-IV inhibitors on gastrointestinal functions in diabetic rats

PKF-275-055 dose-dependently inhibited gastric emptying and small intestinal transit rates, with significance at doses of 1 mg/kg

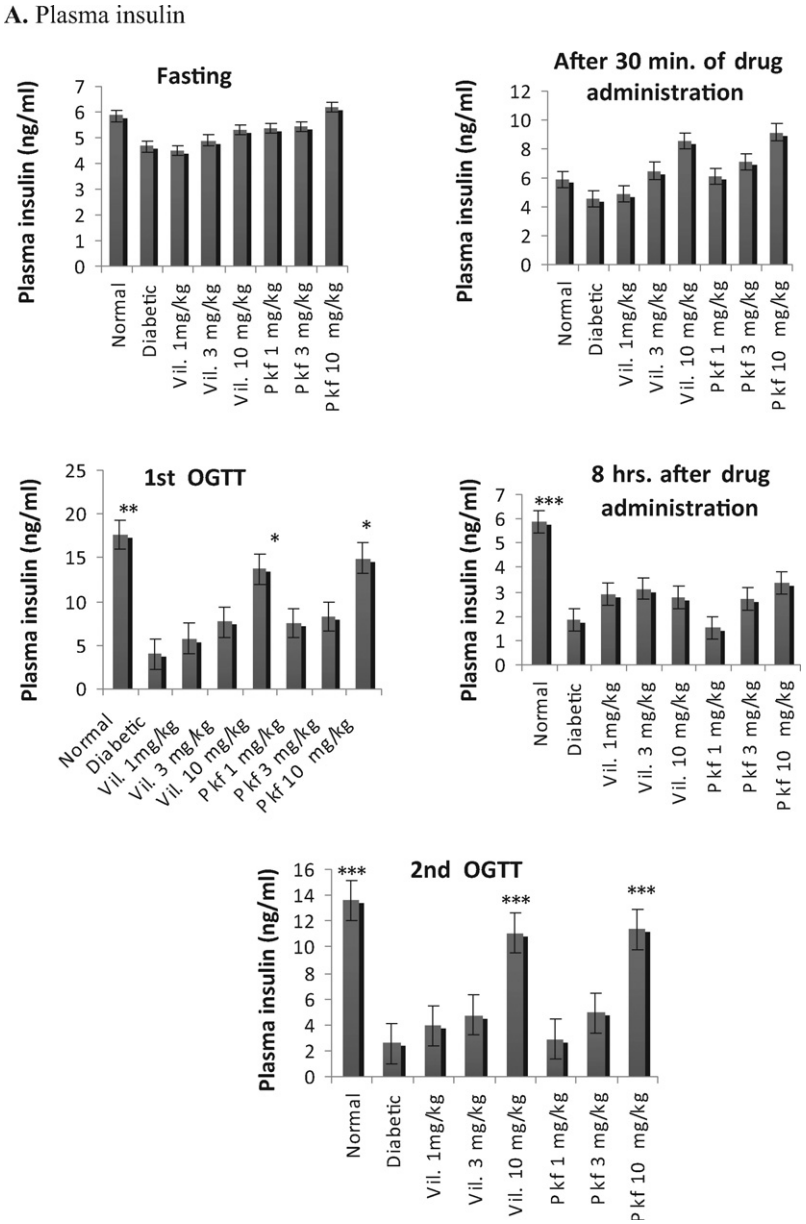

Fig. 5. Effects of vildagliptin and PKF-275-055 on plasma insulin, GLP-1, and DPP-IV levels during fasting, 0 min (pre-OGTT), and 10 min during the oral glucose tolerance test (OGTT) conducted 0.5 h (1st OGTT) and 8 h (2nd OGTT) after drug administration. The values are the means ± S.E.M. from five animals in each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. diabetic group.

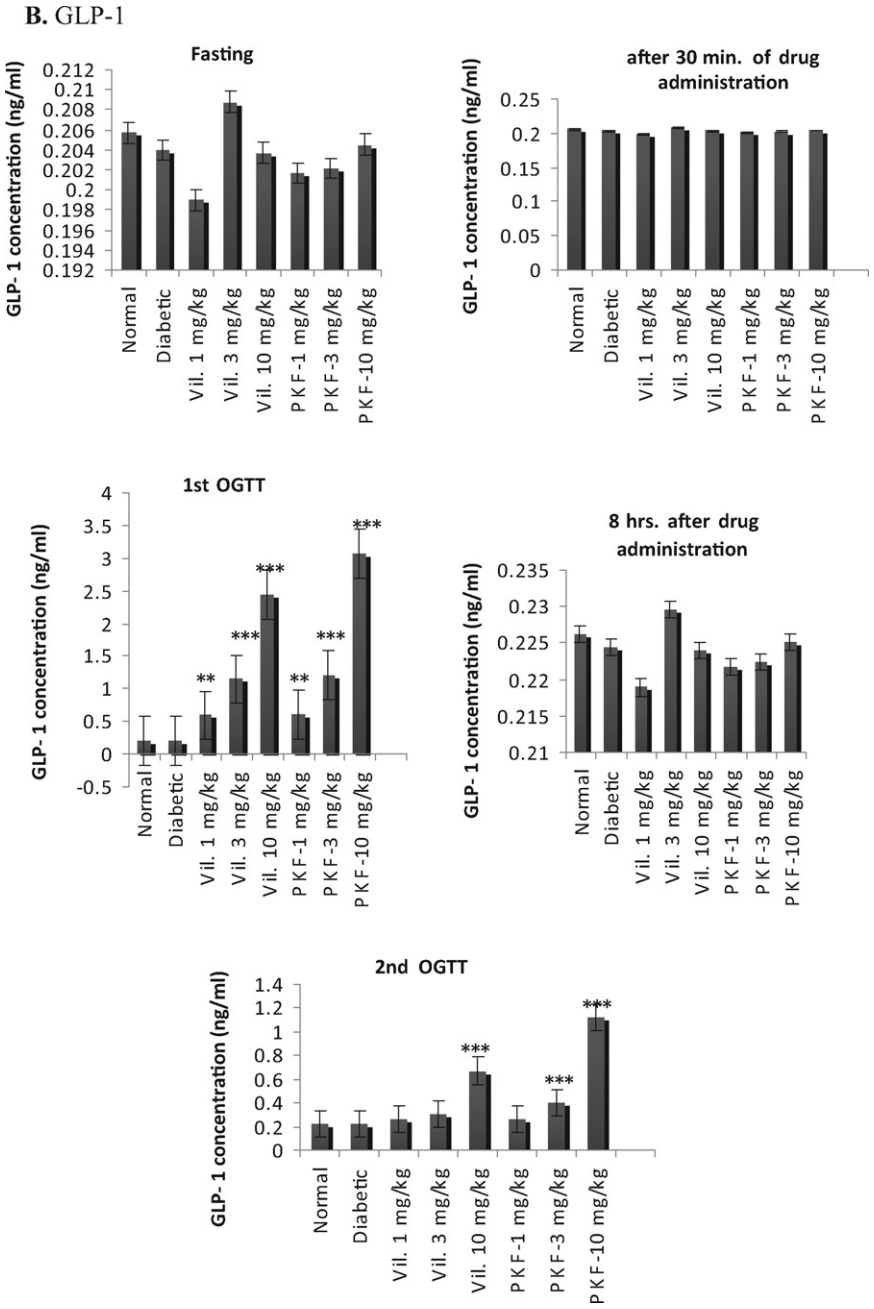

Fig. 5. (*Continued*).

or higher. In contrast, vildagliptin showed dose-dependent inhibition of gastric emptying, but values were not statistically significant; also, vildagliptin did not significantly influence small intestinal transit rates (Fig. 6 and Table 2).

3.6. Effect of chronic daily dosing of DPP-IV inhibitors on glycated hemoglobin (HbA1c), HOMA-Index, and β-cell function in diabetic rats

In streptozotocin induced diabetic rats, vildagliptin (3 and 10 mg/kg; $p < 0.05$) and PKF-275-055 (3 and 10 mg/kg; $p < 0.05$, $p < 0.001$; respectively) significantly inhibited increase in HbA1c level after chronic daily drug administration when compared with diabetic group (Fig. 7 and Table 3). Both vildagliptin and PKF-275-055 dose-dependently inhibited insulin resistance assessed by HOMA-Index, with significance at doses of 1 mg/kg or higher. However, dose dependent improvement in β-cell function showed by both the drug but values were not statistically significant when compared with diabetic group (Fig. 7 and Table 3).

3.7. Effect of chronic daily dosing of DPP-IV inhibitors on histopathologic changes and cell apoptosis in the pancreata of diabetic rats

Histopathologic evaluation of the pancreata of diabetic rats revealed a high frequency of degenerative changes, such as a moderate decrease in the number of insulin-positive granules as well as atrophy, pyknosis, degeneration, and necrosis in the islets. In contrast, degenerative changes occurred at a low frequency in the vildagliptin and PKF-275-055 groups, with only a slight decrease in the number of insulin-positive granules and no marked islet atrophy, degeneration, or necrosis (Fig. 8).

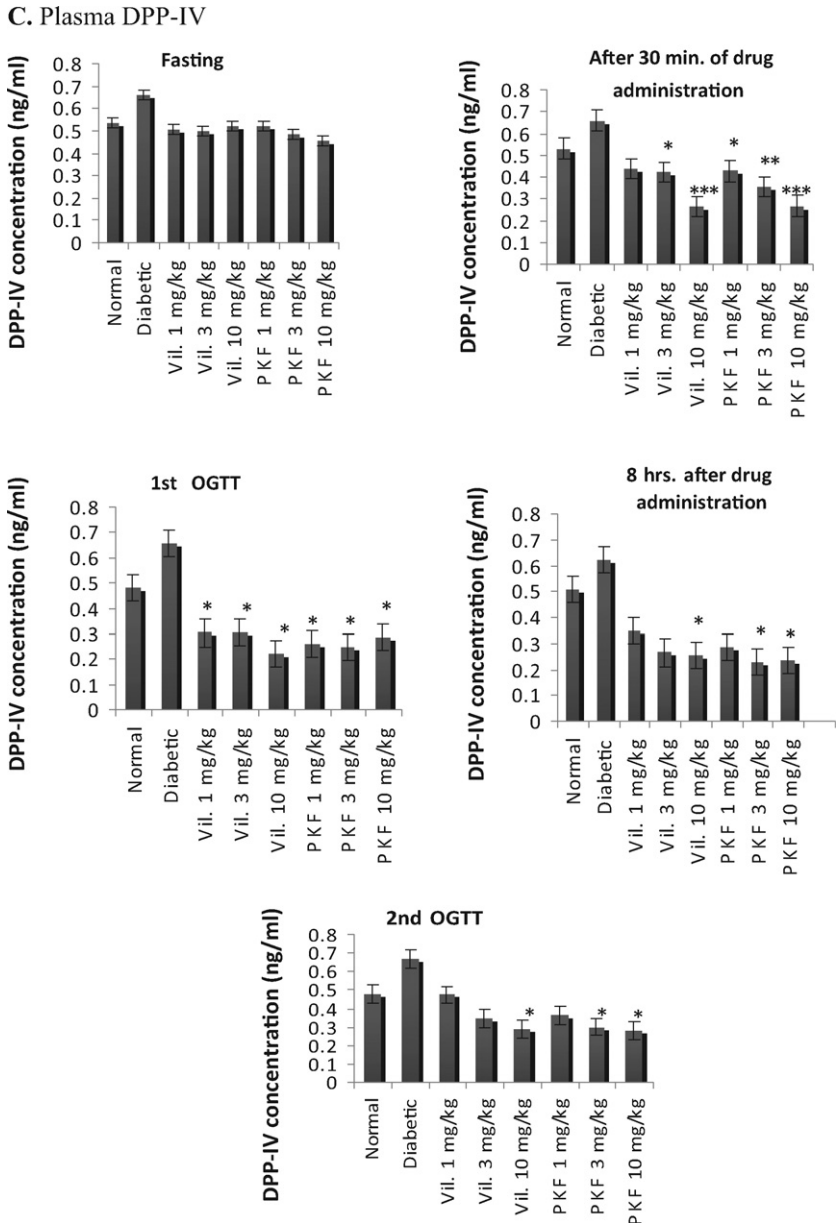

Fig. 5. (*Continued*).

Table 2
Effect of DPP-IV inhibitors on gastrointestinal functions in diabetic rats.

	Gastric emptying rate (%)	Intestinal transit rate (%)
Normal	$37.23 \pm 2.11^{**}$	$50.27 \pm 1.91^{**}$
Diabetic	86.92 ± 12.78	62.34 ± 1.27
Vil. 1 mg/kg	66.59 ± 10.77	58.37 ± 1.85
Vil. 3 mg/kg	58.96 ± 15.97	62.84 ± 3.04
Vil. 10 mg/kg	52.43 ± 4.33	61.4 ± 3.87
PKF.1 mg/kg	$48.72 \pm 7.047^{*}$	$54.12 \pm 0.28^{*}$
PKF.3 mg/kg	$44.06 \pm 7.14^{**}$	$53.83 \pm 0.81^{*}$
PKF.10 mg/kg	$31.44 \pm 1.95^{***}$	$48.63 \pm 1.55^{***}$

The values are the means ± S.E.M. from five animals in each group.
* $p < 0.05$ vs. diabetic group.
** $p < 0.01$ vs. diabetic group.
*** $p < 0.001$ vs. diabetic group.

Table 3
Effect of DPP-IV inhibitors on advance glycation end product (HbA1c), HOMA-Index, and β-cell function.

	HbA1c (%)	HOMA-Index %	β-cell function
Normal	$4.31 \pm 0.3^{***}$	$1.18 \pm 0.14^{***}$	$92.3 \pm 16.76^{***}$
Diabetic	7.43 ± 0.3	2.32 ± 0.17	18.26 ± 1.4
Vil. 1 mg/kg	6.72 ± 0.24	$1.55 \pm 0.12^{*}$	27.91 ± 2.64
Vil. 3 mg/kg	$5.85 \pm 0.66^{*}$	$1.44 \pm 0.14^{**}$	32.6 ± 4.07
Vil. 10 mg/kg	$5.78 \pm 0.38^{*}$	$1.25 \pm 0.27^{**}$	37.3 ± 12.19
PKF.1 mg/kg	6.29 ± 0.34	$1.59 \pm 0.091^{*}$	28.24 ± 1.84
PKF.3 mg/kg	$5.72 \pm 0.33^{*}$	$1.42 \pm 0.16^{**}$	34.8 ± 2.3
PKF.10 mg/kg	$4.98 \pm 0.25^{***}$	$1.48 \pm 0.24^{**}$	40.96 ± 4.49

The values are the means ± S.E.M. from five animals in each group.
* $p < 0.05$ vs. diabetic group.
** $p < 0.01$ vs. diabetic group.
*** $p < 0.001$ vs. diabetic group.

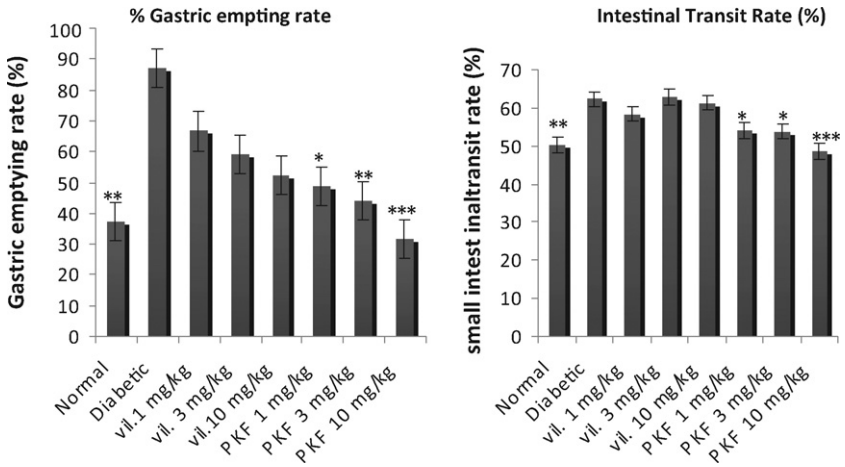

Fig. 6. Effects of vildagliptin and PKF-275-055 on gastrointestinal functions in diabetic rats after drug administration. The values are the means ± S.E.M. from five animals in each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. diabetic group.

Vildagliptin and PKF-275-055 reduces pancreatic cell apoptosis in diabetic treated rats. By contrast, morphological features of apoptosis, including pyknotic nuclei, were readily detectable in pancreatic sections from diabetic rats (Fig. 9).

4. Discussion

DPP-4 inhibitors augment the effects of incretin hormones by prolonging their half-life and represent a new therapeutic

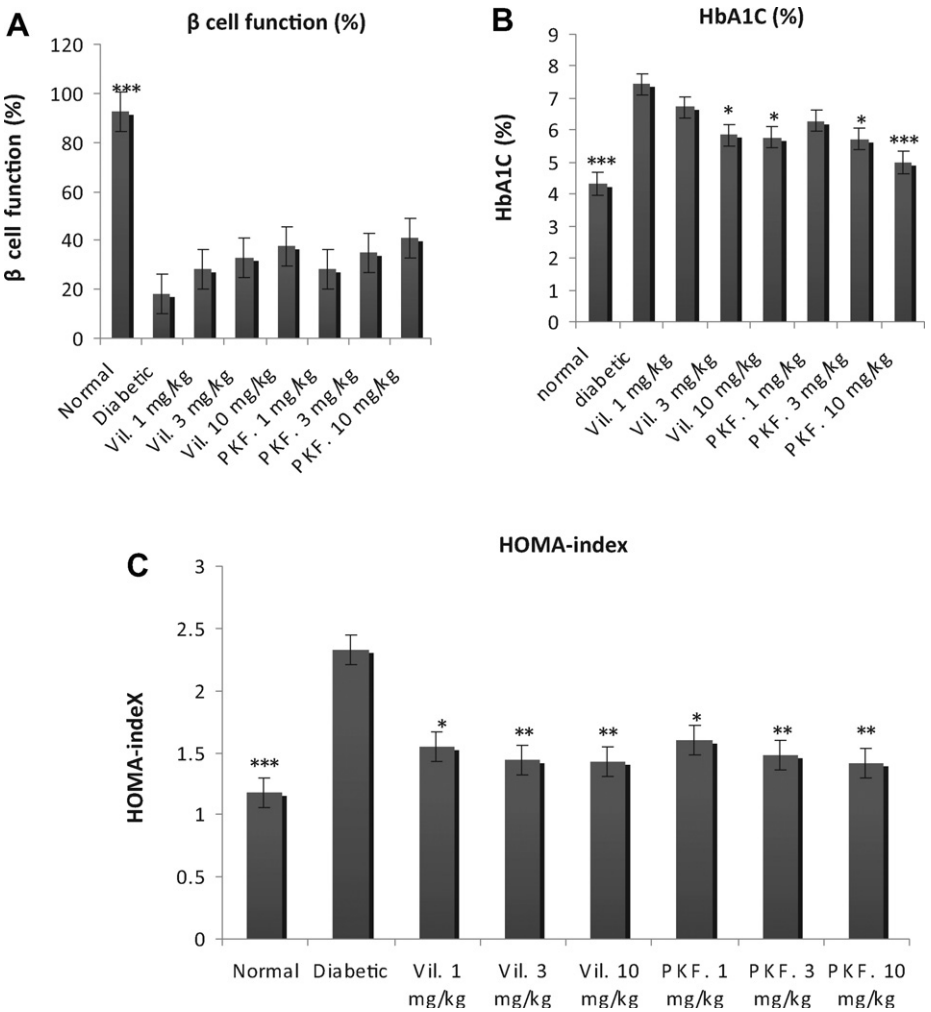

Fig. 7. Effects of vildagliptin and PKF-275-055 on advance glycation end product (HbA1c), HOMA-Index, and β-cell function in diabetic rats after chronic drug administration. The values are the means ± S.E.M. from five animals in each group. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. diabetic group.

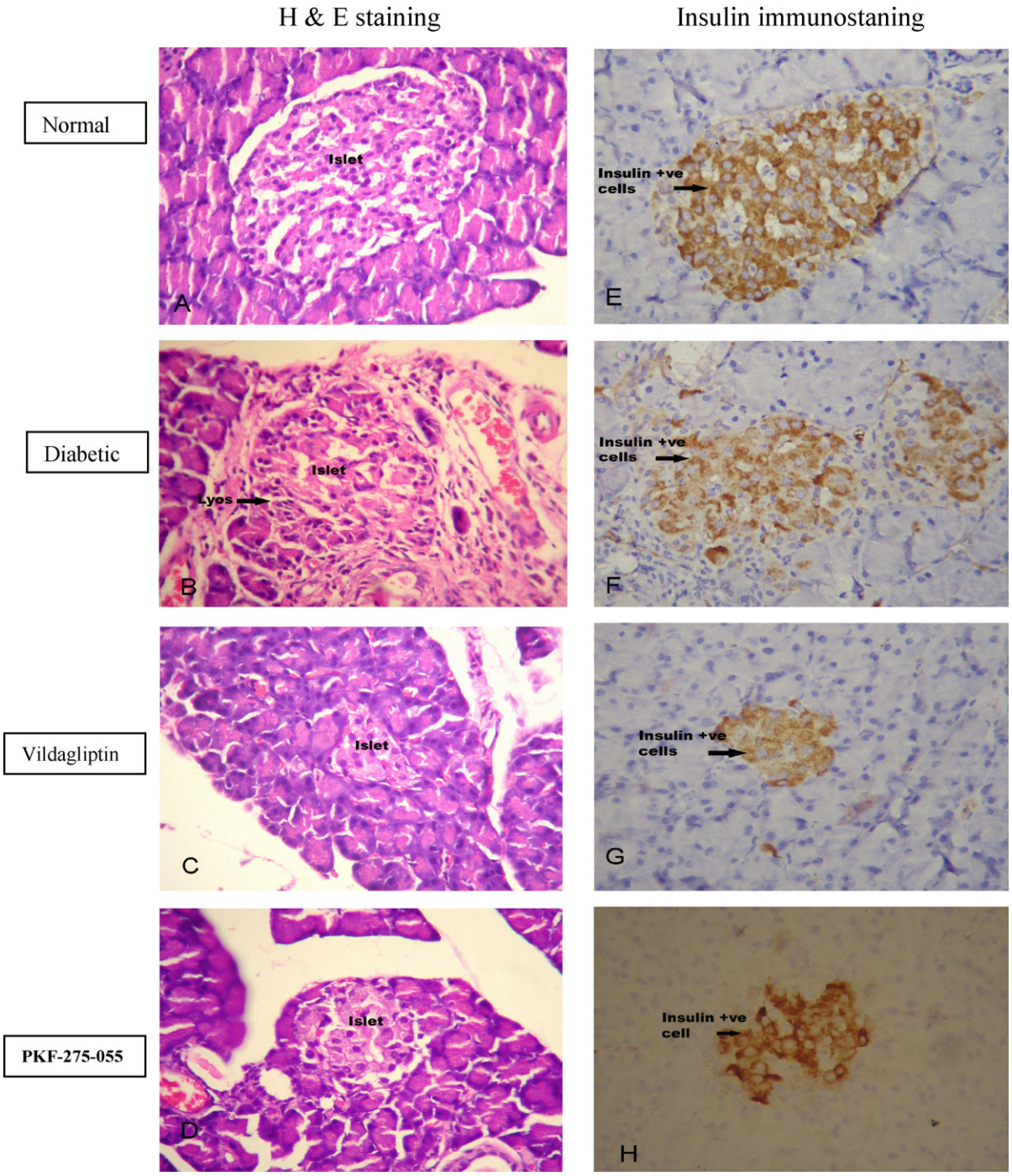

Fig. 8. Effect of chronic daily dosing of vildagliptin and PKF-275-055 on histopathologic changes in the pancreata of diabetic rats. Hematoxylin and eosin staining and anti-insulin antibody immunostaining from (A and E) normal rats, (B and F) vehicle-treated diabetic rats, (C and G) vildagliptin (10 mg/kg)-treated diabetic rats, and (D and H) PKF-275-055 (10 mg/kg)-treated diabetic rats. Original magnification ×400. H&E indicates hematoxylin and eosin.

approach for the treatment of type 2 diabetes [23]. Drawback of vildagliptin includes short half life; inefficiently inhibit gastrointestinal functions, and diabetic complications [15,16]. Therefore, the vildagliptin analogue PKF-275-055 was synthesized as a selective, long-acting inhibitor of dipeptidyl peptidase-4 for the treatment of diabetes and its complications [24]. In the current study, vildagliptin PKF-275-055 was tested after chronic dosing (once a day) in preclinical models of streptozotocin induced diabetes mellitus. Neonatal-STZ wistar model is well characterized model of diabetes mellitus. Neonatal-STZ rats develop persistent diabetes rapidly after 6 weeks of age, and showed diabetes like symptoms such as lack of insulin release in response to glucose, glucose intolerance, raised glycosylated hemoglobin, and depletion of pancreatic insulin store [25–27]. The present study demonstrated that the DPP-IV inhibitors PKF-275-055 have a glucose tolerance-improving effect comparable with or superior to that of vildagliptin, which suggests their usefulness as a therapeutic agent for diabetes mellitus.

In the present study, we investigated the antihyperglycemic effects of PKF-275-055 in streptozotocin induced diabetic rats, which exhibited a mild decline in glucose tolerance due to loss of early-phase insulin secretion [28]. These diabetic rats experienced a approximately 70% decrease in pancreatic insulin content. Furthermore, fasting plasma GLP-1 levels after glucose loading did

not differ between normal and diabetic rats. PKF-275-055 caused significant decreases in the blood glucose levels during both the 1st and 2nd OGTT in diabetic rats. In contrast, vildagliptin had no significant effect during the 2nd OGTT diabetic rats. Furthermore, fasting plasma DPP-IV levels after glucose loading did not differ between normal and diabetic rats. At the dose of 10 mg/kg, both vildagliptin and PKF-275-055 significantly inhibited (>50% inhibition) plasma DPP-IV activity during both the 1st and 2nd OGTT in diabetic rats. In present study, we observed that PKF-275-055 is a selective DPP-IV inhibitor and exhibits a potent and long-acting antihyperglycemic effect based on a glucose-dependent insulinotropic action associated with increases in plasma GLP-1 levels during both the 1st and 2nd OGTT in diabetic rats than vildagliptin. From the chronic study, it was clear that PKF-275-055 rapidly inhibited plasma DPP-4 activity in diabetic rats in a dose-related manner. This action of PKF-275-055 was accompanied by (1) a marked increase in the glucose-stimulated levels of intact GLP-1, (2) enhanced glucose-stimulated insulin levels, and (3) a marked decrease in glucose excursions after an oral glucose challenge. The minimum effective dose of PKF-275-055 to inhibit DPP-4, to augment intact GLP-1, to improve β-cell function, and to reduce glucose excursions was 1 mg/kg, and a dose of 10 mg/kg exerted maximal effects on all parameters. These findings are consistent with those of several earlier studies using other DPP-4 inhibitors in glucose intolerant rodents, including Zucker fatty rats [28,29], high-fat-fed rats [30] and mice [31], streptozotocin-nicotinamide-induced mildly diabetic mice [20] and aged rats [32].

In present study, body weight gain was observed in rats treated for 8 weeks with PKF-275-055 (10 mg/kg) averaged 239.2 ± 5.54 g (≈20%) vildagliptin (10 mg/kg) averaged 234.2 ± 4.36 g (≈17%). This was significantly different from weight gain in the vehicle-treated diabetic rats, which averaged 200.8 ± 5.23 g.

In normal rats, PKF-275-055 and vildagliptin significantly inhibited increases in the blood glucose level during the OGTT. In

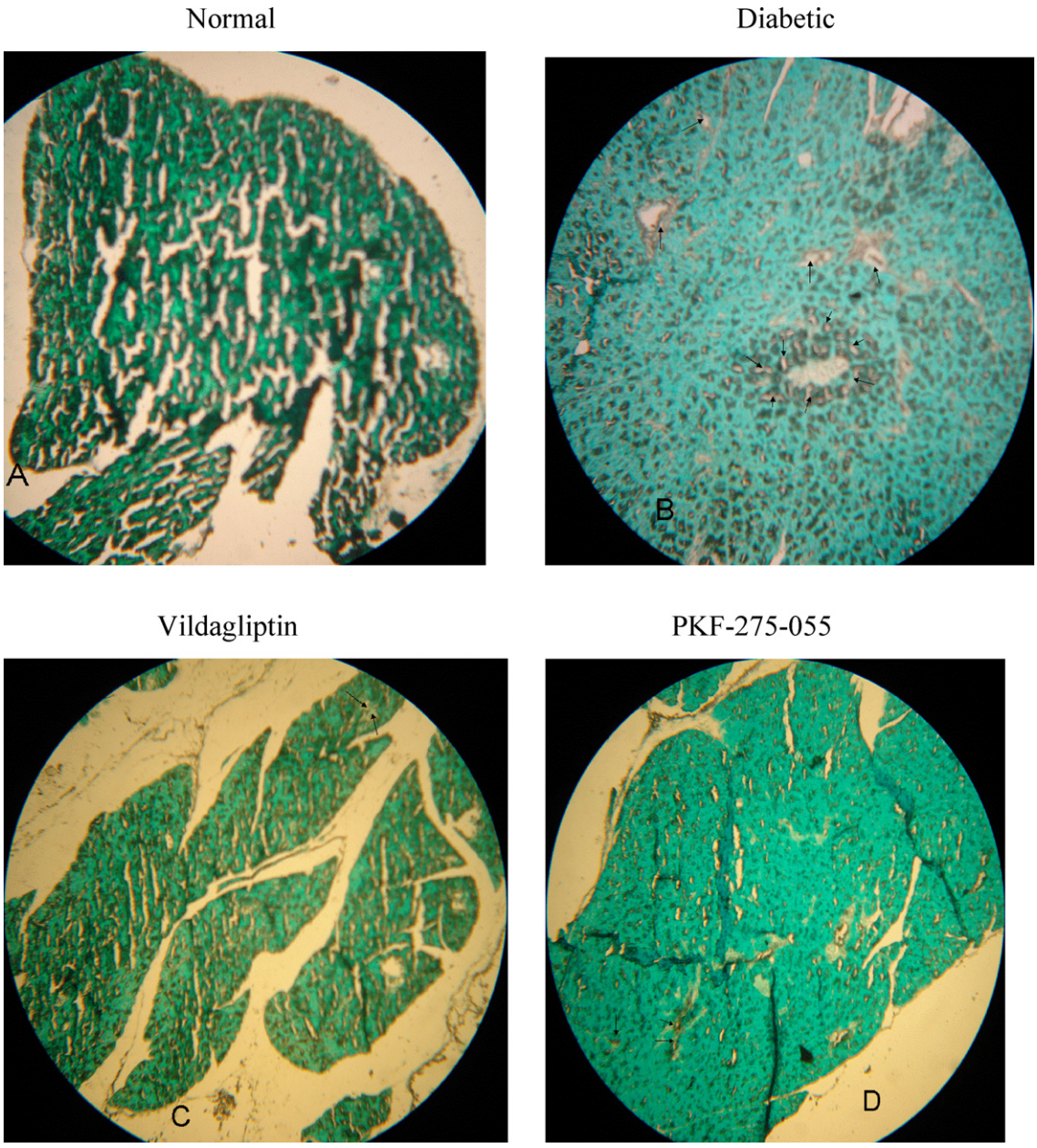

Fig. 9. Effect of chronic daily dosing of vildagliptin and PKF-275-055 on cell apoptosis in the pancreata of diabetic rats. Photomicrograph of cell apoptosis (the arrows denote deoxynucleotidyl-transferase-nick-end-labeling (TUNEL)-positive cells). The TUNEL assay recognizes apoptosis was performed on pancreatic sections of (A) normal, (B) diabetic, (C) vildagliptin (10 mg/kg), and (D) PKF-275-055 (10 mg/kg) treated rats.

contrast, both DPP-IV inhibitors had no significant effect on fasting blood glucose levels (unpublished data). Sulfonylureas, strongly inhibit ATP-sensitive K^+ channel activity by binding to the high-affinity sulfonylurea receptors in pancreatic β-cells, which stimulates insulin secretion glucose-independently. Hypoglycemia has been reported as a side effect with the use of sulfonylureas in diabetic patients [33]. But, DPP-IV inhibitors has no effect on fasting blood glucose levels, there should be no risk of hypoglycemia, which, unlike the existing insulin secretagogue sulfonylurea, makes it safe for use as an antihyperglycemic.

Another incretin, GIP, is secreted from K cells in the duodenum and jejunum in response to oral ingestion of nutrients [34]. Like GLP-1, GIP potentiates glucose-stimulated insulin release, and is degraded by DPP-IV to a biologically inactive form [GIP (3–42)] [35]. It has been reported that DPP-IV inhibitors increased the plasma insulin level and decreased the postprandial blood glucose level in both GLP-1 receptor deficient mice and GIP receptor-deficient mice [36]. However, in double GLP-1 and GIP receptor-deficient mice, the DPP-IV inhibitors had no postprandial blood glucose-lowering effect. These results suggest that both GLP-1 and GIP contribute to the improvement in glucose tolerance elicited by DPP-IV inhibitors. Although the effects of PKF-275-055 on plasma GIP levels were not investigated in this study, GIP may contribute to the antihyperglycemic efficacy of PKF-275-055 in diabetic rats.

In addition to potentiating the effects of GLP-1 and GIP, DPP-IV inhibitors may also prolong the actions of other peptide hormones, such as neuropeptide Y, substance P and growth hormone-releasing hormone, as well as chemokines [37]. Therefore, potential side effects associated with the reduced degradation of other peptide hormones and chemokines need to be considered. However, animals lacking DPP-IV consistently display healthy phenotypes [38,39], and to date, no serious side effects due to DPP-IV inhibition have been reported in clinical studies [40,41]. Hence, DPP-IV inhibition may not produce undesirable changes in downstream biological pathways, despite altering the relative levels of intact-to-cleaved peptide substrates. In addition, PKF-275-055 showed significant inhibitory activity for DPP-IV than vildagliptin, but not showed any undesirable effects in 8 weeks chronic treatment.

GLP-1 not only stimulates insulin secretion glucose dependently, but also acts as a physiological mediator for various gastrointestinal functions. Recent studies revealed that, in addition to the incretin effect, exogenous GLP-1 or GLP-1 derivatives also caused a delay in gastric emptying and intestinal transit rates, which was considered to be partially responsible for the inhibition of postprandial hyperglycemia [11,42]. From the present study, we observed delay in gastric emptying when the incretin effect was induced through increased endogenous GLP-1 levels after administration of a DPP-IV inhibitor [14]. In this study, PKF-275-055 dose-dependently inhibited gastric emptying and small intestinal transit rates, with significance at doses of 1 mg/kg or higher. In contrast, vildagliptin also showed dose-dependent inhibition of gastric emptying, but values were not statistically significant; also, vildagliptin did not significantly influence small intestinal transit rates. In meta-analysis of randomize clinical trials, Monami et al. reported nausea, headache, and gastrointestinal disturbances resulting from a DPP-IV inhibitor [43]. This may be due to a delay in gastric emptying and reduced small intestinal transit, which leads to a feeling of fullness.

Immunological staining of the pancreata of the animals treated with both the DPP-IV inhibitors showed that these animals had more intense insulin staining and fewer vacuoles in their islets than their diabetic controls (Fig. 8). Furthermore, vildagliptin and PKF-275-055 reduces pancreatic cell apoptosis in diabetic treated rats (Fig. 9). Both PKF-275-055 and vildagliptin treatment for 8 weeks significantly unregulated β-cell insulin content. This effect to increase insulin content was also seen in *P. obesus* treated with the GLP-1 analogue S 23521 and has also been observed in other animal models [44]. Studies in rodent islets have shown that GLP-1 acts to directly regulate the insulin gene and upregulates genes involved in insulin biosynthesis [45–47] and although we did not measure these parameters, we believe this was probably an important aspect of the improved β-cell function in these animals.

Vildagliptin has been shown to lower blood glucose and HbA1c in human studies [41,48] and decrease plasma glucose and increase plasma insulin in rodents [49–51]. In current study, both vildagliptin and PKF-275-055 at the dose of 3 and 10 mg/kg significantly inhibited increase in HbA1c level and HOMA-Index compared with diabetic group. Furthermore, dose dependent improvement in β-cell function showed by both the drug but values were not statistically significant as compared with diabetic group. Vildagliptin, as well as other DPP-IV inhibitors, has been shown to increase proliferation, β-cell mass (BCM), and pancreatic insulin content and decrease apoptosis [52–54,7–9]. In the present study, Eight week treatment with PKF-275-055 showed effects on proliferation, BCM, and pancreatic insulin content.

In conclusion, the present study shows that PKF-275-055 is a selective DPP-IV inhibitor with potent antihyperglycemic activity and no effect on fasting blood glucose levels. Furthermore, it has beneficial effect on β-cell mass recovery and glucose homeostatis. The results suggest the usefulness of PKF-275-055 for further development as a therapeutic agent for impaired glucose tolerance and diabetes mellitus.

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