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Original article

DPP4 inhibitor induces beta cell regeneration and DDR-1 protein expression as an endocrine progenitor cell marker in neonatal STZ-diabetic rats

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ABSTRACT

Background: We aim to investigate the effects of dipeptidyl-peptidase-4 inhibitor (Vildagliptin-VG) on DDR-1 as a marker for endocrine progenitor cells, β-cell regeneration, and apoptosis in neonatal streptozotocin (n2-STZ) diabetics.
Methods: Neonatal rats were divided into two main groups as short- and long-term treatment, each consisted of four groups; (1) Control, (2) n2-STZ diabetic (single dose of 100 mg/kg STZ at 2nd day of birth), (3) n2-STZ + VG (60 mg/kg/day VG orally; for 8 and 28 days), (4) VG (60 mg/kg/day orally; for 8 and 28 days). Blood glucose levels and body weights were measured, and the tissue sections were immunostained using insulin, glucagon, somatostatin, PCNA, Pdx-1 and DDR-1 antibodies. The TUNEL method was used for apoptosis.
Results: The number of β cells in islets of the n2-STZ + VG group increased compared to the n2-STZ group; insulin (+) cells were observed individually or as small clusters in exocrine tissue, between pancreatic duct epithelial cells, and around the ducts. The number of Pdx-1 and DDR-1 positive cells in islet and extra-islet pancreas tissue was elevated as a result of VG application compared to the STZ diabetic group; the number of double positive cells for DDR-1 and insulin increased in n2-STZ + VG rats.
Conclusion: We showed that vildagliptin promotes β cell neogenesis and regeneration, stimulates DDR-1 expression as an endocrine cell progenitor marker, suppresses apoptosis, induces islet cell proliferation and rearranges islet morphology in the n2-STZ diabetes model.

Introduction

Diabetes mellitus is a systemic, chronic metabolic disease that develops due to a decrease in the number of pancreatic β cells and by the impairment of their functions. Various factors including insulin resistance, glucotoxicity, hyperinsulinemia, lipotoxicity, endoplasmic reticulum stress and hyperglycemia in Type 1 and Type 2 diabetes cause damage and loss of pancreatic β cells [1].

The treatment methods for diabetes have generally targeted insulin deficiency or insulin resistance. The new strategies developed for disease treatment include models inducing pancreatic β cell regeneration or differentiation of progenitor cells into β cells [2]. It has been suggested that the pancreatic islet endocrine cells may originate from specific progenitor cells exhibiting a particular type of endocrine characteristic [3].

Glucagon-like peptide-1 (GLP-1), an incretin hormone, induces insulin release, inhibits glucagon secretion, and regulates plasma glucose concentration [4]. GLP-1 also regulates the functions of various genes in islet β cells and increases β cell mass by inducing the growth and differentiation of these cells [5]. Endogenous DPP enzyme cleaves GLP-1, resulting in an alteration in the glycemic index. DPP inhibitors have been associated with the regulation of increased blood insulin levels and regeneration of β cells in the pancreas [6]. Reports suggest that vildagliptin (VG), a DPP4 inhibitor used in the treatment of Type 2 diabetes, inhibits the degradation of GLP-1 and increases the plasma glucose depending on insulin secretion, leading to an increase in plasma GLP-1, and therefore significantly regulates plasma glucose levels [7].

One of the most important β cell markers is the pancreatic duodenal homeobox-1 (Pdx-1), a transcription factor which has a

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crucial role in pancreatic development and β cell differentiation [8]. The expression of Pdx-1, which regulates the activation of many specific β cell genes, has been detected predominantly in adult β cells [9]. It has been suggested that pancreatic islet cells originate from intra-islet and extra-islet progenitor cells [3,10–12]. DDR-1, a member of the DDR (Discoidin domain receptors) family, has been suggested as a bio-surface marker for endocrine progenitor cells, specifically in the pancreatic islets [12]. DDR-1 is a transmembrane protein located on the plasma membrane that plays a role in cell proliferation, migration, differentiation, and remodeling of the extracellular matrix by binding with collagen molecules [13]. DDR-1 expression has been identified primarily in the kidney, lung, gastrointestinal system and brain, as well as in fibroblasts and bone cells [14]. Furthermore, DDR-1 is not expressed in the pancreas of adult mice and humans. However DDR-1 expression was observed during the embryonic stage in the pancreas of mice, as co-located with Pdx-1 positive and Ngn3 positive progenitor cells, suggesting a role for DDR-1 in endocrine cell differentiation [12].

Until now, the distribution of DDR-1 expression in the endocrine and exocrine pancreas has not been defined under diabetic conditions. The present study investigates the effects of VG on β cell regeneration and DDR-1 expression, an accepted marker for endocrine progenitor cells, and attempts to show a possible induction of β cell progenitors by VG, as well as its effects on islet morphology and apoptosis in a neonatal rat STZ diabetes model.

Material and methods

Animals

We followed the principles of laboratory animal care and national laws on animal use in this present study which was approved by the Animal Welfare and Ethics Committee of Istanbul University. The Experimental Animal Breeding and Research Laboratory of Istanbul University supplied the 48 neonatal Wistar albino rats used in this study. During the experiment, the short-term group's litter was fed *via* breastfeeding up to the 10th day. The long-term group's litter was fed *ad libitum* from the beginning of the 22ndday. The following experimental protocol was applied to the neonatal STZ diabetes model.

Experimental design

Neonatal rats (n = 48) were assigned to an experimental group on the 2nd day after their birth. Experimental animals were divided into two main groups according to short-term and long-term drug application (8 days and 28 days, respectively), including 4 subgroups in each: (1) Neonatal control group (C) physiological saline applied. (2) Neonatal STZ diabetic group (n2-STZ); at the 2nd day of birth, a single dose of 100 mg/kg STZ dissolved in physiological saline was injected *ip* [15]. (3) Neonatal STZ-diabetic VG treatment group (n2-STZ + VG): 60 mg/kg/day dose of VG dissolved in physiological saline injected orally to STZ-treated rats beginning on the 3rd day of birth (4) VG control group: only 60 mg/kg/day dose of VG was applied orally beginning on the 3rd day of birth.

Applications for all groups were terminated at the 10th and 30th days after birth. VG was kindly provided by Novartis Pharma K.K. (Basel, Switzerland) and was prepared as an emulsion in distilled water. In this study, STZ application was performed on the second day. On the 3rd day (STZ injection after 24 h), animals with blood glucose >200 mg/dl were taken into experimental groups, and VG injections were performed. Blood glucose measurements were repeated on 4th and 10th days.

Blood glucose and body weight

A glucometer (Accu-check, Roche, Mannheim, Germany) was used to measure fasting blood glucose levels from one drop of blood collected from rat tails on the 2nd. day (before STZ application), and on days 3, 4, 10, 20 and 30; body weights were also measured on those same days.

Tissue preparation

At the end of the experiment, pancreatic tissue samples were collected from the rats under Ketamine HCl (50 mg/kg, *ip)* and Xylazine HCL (20 mg/ml) anesthesia. The pancreas biopsies were fixed in 10% neutral buffered formalin, followed by embedding in paraffin. Five μm thick sections were placed onto adhesive slides, then deparaffinized and rehydrated. Immunohistochemistry with insulin, glucagon, somatostatin, Pdx-1, PCNA and DDR-1 antibodies was applied to pancreatic tissue sections, in addition to the TUNEL method to detect apoptosis.

Immunohistochemistry

Antibodies

Insulin, (Sigma, I-2018; 1:1500 dilution), Glucagon, (Zymed; 18-0064; 1:50), Somatostatin, (Zymed, 18-0078; 1:75), Pdx-1 (Pancreatic Duodenal Homeobox-1) (a gift from Prof.Dr. CV Wright, Vanderbilt University, USA; 1:200), DDR-1 (Discoidin Domain Receptor-1) (Santa Cruz sc-532; 1:200), and PCNA (NeoMarkers MS-106-P; 1:200) were utilized for immunohistochemistry. Double-immunohistochemistry was performed using DDR-1 and insulin antibodies.

Immunostaining

The streptavidin-biotin-peroxidase immunostaining method was applied for immunohistochemical analysis. The Histostain Plus Bulk Kit (Life Technologies-Invitrogen) was used as the staining kit; steps of the procedure recommended in the protocol were modified according to the antibodies used. Antigen retrieval for somatostatin and glucagon antibodies was performed in 10 mM citrate buffer (pH 6.0) for 20 min using a microwave oven. Enzymatic antigen retrieval was performed using Proteinase K (20 μg/ml) for DDR-1, a membrane protein. 1% PBS Triton X-100 washing solution was prepared, and endogenous peroxidase activity was blocked by 3% H_2O_2 in methanol. For enzymatic color reaction, 3-amino,9-ethyl-carbazole (AEC) substrate was used and subsequently counterstained with hematoxylin.

Double immunohistochemistry

The pancreas tissue sections were stained with double immunohistochemistry for insulin and DDR-1 antibodies. A double immunohistochemistry kit, ZytoChem Plus Double Stain Polymer Kit, POLDS-006, was used and the recommended steps of the staining protocol were applied. Fast Red substrate kit (Zytomed System, Red055, Germany) and DAB substrate kit (Zytomed System, DAB057, Germany) were used for color reaction in the double immunohistochemistry method; sections were counter-stained with methyl green, then evaluated under a light microscope.

The protocol was applied to the negative control slide to control specificity of immunostaining using PBS instead of the primary antibody.

Morphometric analysis

Morphometric analysis was performed onsections using a Leica DM 2500 light microscope attached to a Leica DFC280 digital

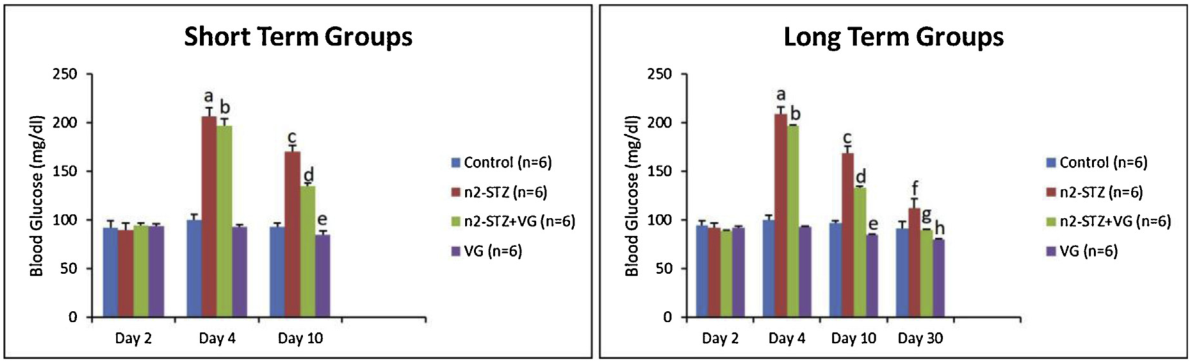


Fig. 1. Comparison of the blood glucose (mg/dl) for all groups in the short-term and the long-term. $^{a,c}p < 0.001$, $^{e}p < 0.05$ compare to the control group; $^{b,d}p < 0.001$ compare to the n2-STZ group in the short-term group. $^{a,c,e,f}p < 0.001$, $^{h}p < 0.05$ compare to the control group; $^{b,d,g}p < 0.001$ compare to the n2-STZ group in the long-term groups.

camera system (Leica Microsystems, Wetzlar, Germany) at a magnification of 200 × . Automatic image analysis of pancreatic sections for calculation of tissue areas was performed with Leica LAS version morphometric analysis software. The size of the islets was classified according to islet size as large (>10,000 μm2), medium (5000–10,000 μm2) and small (<5000 μm2) islets in the sections, then the percentage for all groups was calculated [15].

The percentage of immunoreactive islet cells for either (insulin or glucagon was calculated ((Total positive cells area / Total islets area) x 100). The immunostained PCNA, DDR-1, Pdx-1, and somatostatin cells were counted using a light microscope ((Total immunopositive cell numbers / Total islets cell numbers) × 100). Cell counts were performed using a 400x magnification in different fields. At least 10-islets were evaluated in each tissue section from every animal in the experimental groups [15].

In situ *DNA end labeling method (TUNEL)*

Detection of DNA fragmentation *in situ* was visualized with the use of the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, USA), and the TUNEL method was performed for detection of apoptotic cells. The tissue sections were incubated with proteinase K (20 μg/ml, in PBS) (Sigma–Aldrich, St. Louis, USA). The following procedure was applied. 3% H_2O_2 in PBS was used for endogenous peroxidase inhibition. The tissue sections were incubated with 1X equilibration buffer at room temperature (RT), and the digoxigenin-labeled dNTPs were incubated with terminal deoxynucleotidyl transferase (Tdt) enzyme for 1-h at 37 °C. Then the sections were washed in stop/wash buffer for 10 min at RT and incubated with anti-digoxigenin peroxidase antibody at RT (30 min). Diaminobenzidine (DAB) was applied to the sections as a peroxidase substrate. Staining was evaluated under the light microscope after the sections were counterstained with methyl green. The rat mammary gland sections (Millipore, S7115) were used as positive control for the staining specificity, and also distilled water was used instead of Tdt enzyme as a negative control.

Apoptotic index

The labeled apoptotic cells in tissue were counted under 400x magnification, using all islets for each section. Apoptotic index was calculated according to the following formula: Apoptotic index = (Apoptotic cell numbers / Total cell numbers) x 100 [16].

Statistical analysis

All measurements and counts were analyzed statistically by using Graphpad 5.0 software program. One-way ANOVA, *t*-test, Kruskal Wallis, and Mann-Whitney U testing were used as statistical tests. $p < 0.05$ values were accepted as significant, $p < 0.01$ values were accepted as highly significant and $p < 0.001$ was accepted as extremely significant.

Results

Blood glucose levels and body weights

A significant increase in blood glucose levels in the short-term and long-term experimental groups was detected following STZ application in the diabetic rats compared to the control group ($p < 0.001$), while a significant decrease in the n2-STZ + VG group was noted compared to the STZ applied group ($p < 0.001$). In both the short and long-term, the VG group's blood glucose levels decreased significantly compared with the control groups ($p < 0.05$) (Fig. 1). In the short-term group, body weights of the n2- STZ + VG and VG groups showed a significant decrease compared with the n2-STZ group ($p < 0.001$). In the long-term groups, body weights of the n2-STZ + VG group decreased compared with the control group ($p < 0.001$) (Table1).

Size of islets

Morphometric analysis software was used to measure islet sizes and calculate their percentage in the tissue section. The distribution of the three groups of islets: small (<5000 μm2), medium (5000–10,000 μm2) and large (>10,000 μm2) for the short and long-term groups can be seen in Table2.

Immunohistochemistry

Insulin

In the short-term group, the area containing insulin-positive cells was reduced in the n2-STZ group compared with the control

Table 1
Comparison of the body weights (BWs) for all groups in the short -term and the long -term.

Days	BWs (g)			
	Control	n2-STZ	n2-STZ + VG	VG
Short term				
2	9.66 ± 0.408	9.50 ± 0.447	9.50 ± 0.447	9.50 ± 0.447
10	18.50 ± 0.447	16.08 ± 0.376*	10.58 ± 0.584**	11.25 ± 0.689***
Long term				
2	9.58 ± 0.37	9.50 ± 0.44	9.50 ± 0.447	9.50 ± 0.447
10	18.68 ± 0.55	16.41 ± 0.37*	10.66 ± 0.510**	11.33 ± 0.75***
30	40.58 ± 0.49	50.58 ± 2.87^{a}	49.00 ± 0.836^{c}	54.50 ± 1.04^{b}

$^{*,***}p < 0.001$ *versus* control group; $^{**}p < 0.001$ *versus* n2-STZ group; $^{***,\ a,b,c}p < 0.001$ *versus* control group.

Table 2
Variation in size of the pancreatic islets (%) in all groups.

Islets sizes	Pancreatic Islets (%)			
	Control	n2-STZ	n2-STZ + VG	VG
Short-term				
Small	15	84	47	33
Medium	54	13	44	12
Large	31	3	9	55
Long-term				
Small	4	38	39	20
Medium	14	42	14	15
Large	83	20	57	65

group. Also, the islets with insulin-positive cells were smaller in the STZ applied diabetic group than the control group. In the n2-STZ group, islet β cell numbers were less than the control group ($p < 0.001$). Moreover, small islets including with highly insulin (+) cells were seen besides the weakly insulin-positive cell islets. Insulin immunopositive cell clusters and newly formed small islets were found in exocrine tissue and close to the pancreatic ducts. The area covered by insulin (+) cells and the size of islets in the n2-STZ + VG group increased in comparison with the n2-STZ group ($p < 0.05$). Reorganization of intra-islet β cells due to VG application was observed in the n2-STZ group, similar to the control group. In this group, insulin (+) cells were seen in exocrine tissue scattered individually or as small clusters, as well as around the duct and in the epithelial cells of the pancreatic duct. Largerhans islet sizes and insulin (+) areas were noted in the group with VG only applied than the control group ($p < 0.05$). Also, more insulin (+) cells were observed individually or as small clusters in exocrine tissue in comparison with the control group. Additionally, insulin (+) cells were detected as individually in between duct epithelial cells or in the exocrine pancreas. The results obtained in long-term experimental groups were similar in terms of number and distribution of insulin immunopositive cells to the short-term findings. All results are shown in Fig. 2, Table 3A and B.

Pdx-1

Cells with nuclei stained by Pdx-1 antibody were assessed as originally Pdx-1 nuclear positive cells (N+) in pancreatic tissue sections, in addition to cells with stained cytoplasm accepted as cytoplasmic positive (C+). In both the short and long-term STZ groups, the number of Pdx-1 (+) cells markedly declined compared to the control group ($p < 0.001$). An increase in the numbers of (+) cells in the n2-STZ + VG group was detected compared to the n2-STZ group ($p < 0.001$). Pdx-1(+)cell numbers declined in the short-term VG group but elevated in the long-term VG group compared to the control. The C + cell number for the VG applied group in the short-term was similar to the control group, but the C + cell number decreased in comparison with the control group ($p < 0.01$) in the long-term. For both the short and long-term

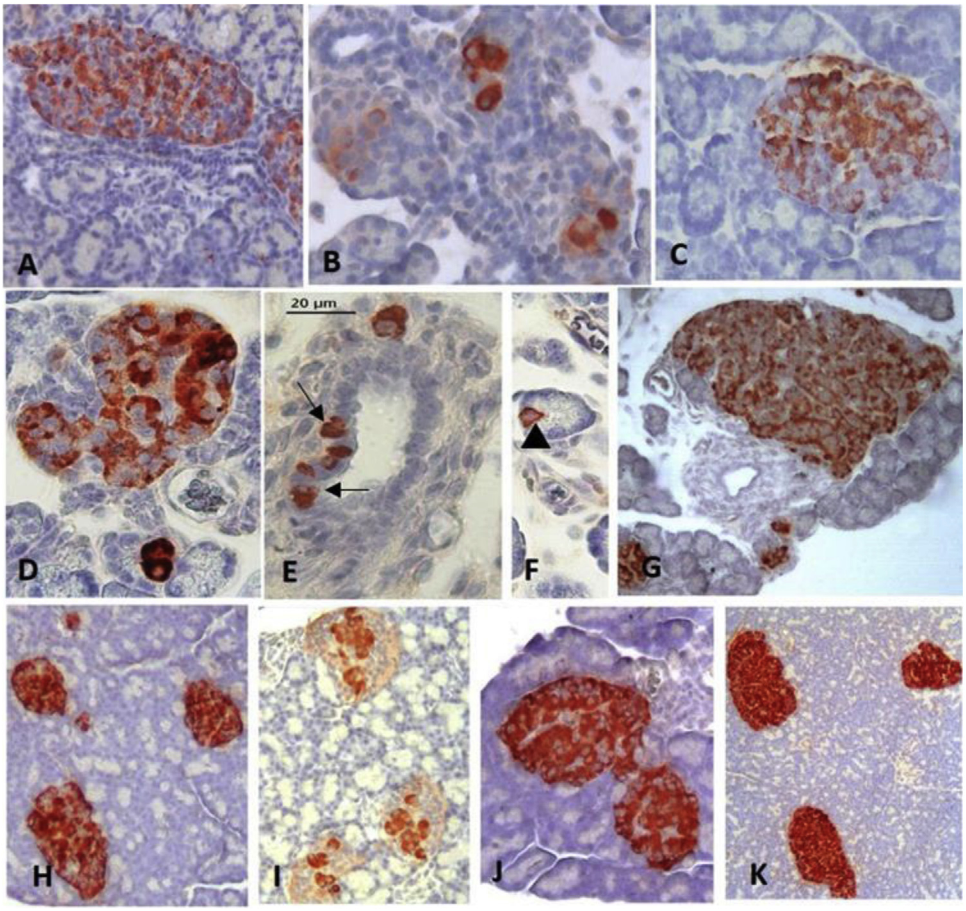


Fig. 2. Immunohistochemistry for insulin in the short-term (A–F) and the long-term (H–K) groups. (A) Control group; the islets of the short-term are filled with insulin immunopositive cells. (B) In the n2-STZ group, decreased insulin positive cells are seen in the islets. (C–F) In the n2-STZ + VG group; the number of insulin positive cells is increased compared to the STZ group, and immunopositive cells are observed in the duct epithelium (↑) and exocrine (▲) tissues. (G) VG group; Insulin positive cells are filled in the islet. The long term groups are shown similar pattern to the short-term. H; Control, I; n2-STZ group, J; n2-STZ + VG and K; VG groups. Immunostaining: Streptavidin-biotin-peroxidase, Counterstaining: Hematoxylin, Magnification: A,C,G–J: 200x ; B,D–F: 400x ; K: 100x.

Table 3
Comparison of areas covered by insulin (+) cells in islets (%) (A), and the number of insulin immunopositive cell (B).

A	Areas covered by insulin (+) cells in islets (%).			
	Control	n2-STZ	n2-STZ + VG	VG
Short term	81.175 ± 2.312	62.597 ± 2.413^{a}	69.370 ± 1.641^{b}	88.500 ± 1.517^{c}
Long term	90.877 ± 1.110	81.782 ± 3.123^{d}	88.363 ± 5.079^{e}	93.767 ± 2.248^{f}

B	Number of insulin immunopositive cell in pancreatic tissue			
	Control	n2-STZ	n2-STZ + VG	VG
Short term				
Exocrine	11.833 ± 1.169	15.833 ± 1.470^{a}	$24.833 \pm 0.9832^{b,k}$	16.833 ± 0.7528^{c}
Duct	2.333 ± 0.516	5.333 ± 0.817^{d}	$3.500 \pm 0.5477^{e,m}$	1.667 ± 0.5164
Small islets	5.667 ± 0.515	12.000 ± 0.632^{f}	$16.833 \pm 0.7528^{g,n}$	19.333 ± 1.2110^{h}
Long term				
Exocrine	11.000 ± 0.89	15.160 ± 0.98^{a}	$40.160 \pm 1.32^{b,*}$	16.000 ± 0.63^{c}
Duct	0.340 ± 0.51	2.500 ± 0.54^{d}	0.505 ± 0.55^{e}	2.833 ± 1.16^{f}
Small islets	8.833 ± 0.75	24.667 ± 0.81^{g}	$37.500 \pm 1.04^{h,k}$	11.000 ± 1.78^{j}

$^{a,d}p < 0.001$ *versus* control; $^{b}p < 0.001$, *versus* n2-STZ; ^{c}p < 0.001, *versus* control ; $^{e}p < 0.05$, *versus* n2-STZ, $^{f}p < 0.05$, *versus* control.
$^{a,d,f}p < 0.001$ *versus* control; $^{b,e,g}p < 0.001$, *versus* n2-STZ; $^{c,h,k,n}p < 0.001$, *versus* control; $^{m}p < 0.05$ *versus* control (B, Short Term)
$^{a,d,g}p < 0.001$ *versus* control; $^{b,e,h}p < 0.001$, *versus* n2-STZ; $^{c,f,*,k}p < 0.001$, *versus* control; $^{j}p < 0.05$ *versus* control (B, Long Term).

groups, all Pdx-1 (+) cells were shown to disperse individually and/or as small clusters inside exocrine tissue of the n2- STZ and n2-STZ + VG groups, as well as to locate to the periphery or in between pancreatic duct epithelial cells. Pdx-1 (+) cells located individually or as small clusters in exocrine tissue or ductal epithelium were observed more in the short and long-term VG groups than the control group. All results are shown in Fig. 3, Table 4 A and B.

Glucagon and somatostatin

Glucagon and somatostatin-positive cells were located at the islet periphery in the short-term control group. In the n2-STZ group, both cell types were distributed toward the center of the islets. The area covered by glucagon (+) cells and the number of somatostatin (+) cells in the n2-STZ group increased in comparison with the control group ($p < 0.001$). In the n2-STZ + VG group, glucagon and somatostatin cells re-located to the periphery similar to the control group, suggesting amelioration in the morphology of the islets. There were glucagon (+) and somatostatin (+) cells dispersed throughout the exocrine tissue in the n2-STZ + VG group; moreover, the area of the glucagon (+) cells notably reduced compared to the n2-STZ diabetic group ($p < 0.001$). Smaller islets with glucagon-positive cells and a reduced number of somatostatin immunopositive cells were observed in only the VG applied group compared to controls ($p < 0.05$ and $p < 0.001$, respectively) (Fig. 4A, Table 5). In the long-term groups, the distribution and localization of glucagon cells were similar to those of the short-term groups. On the other hand, we detected that somatostatin cells in the long-term VG group increased in comparison to the control group ($p < 0.001$) (Fig. 4B, Table 5).

PCNA

In the short-term n2-STZ + VG and VG groups nuclear stained PCNA (+) cells were shown to increase in comparison with the other groups. In the long-term groups, VG applied STZ diabetic rats had higher immune positivity for PCNA, compared to the control and n2-STZ diabetic groups. A significant increase in the number of PCNA-positive cells was detected in the islets of the VG-applied group only, compared to all the other groups ($p < 0.001$) (Fig. 4, Table 6).

TUNEL

The TUNEL method was used to detect apoptotic cells; cells with stained nuclei were assessed as TUNEL positive cells. The

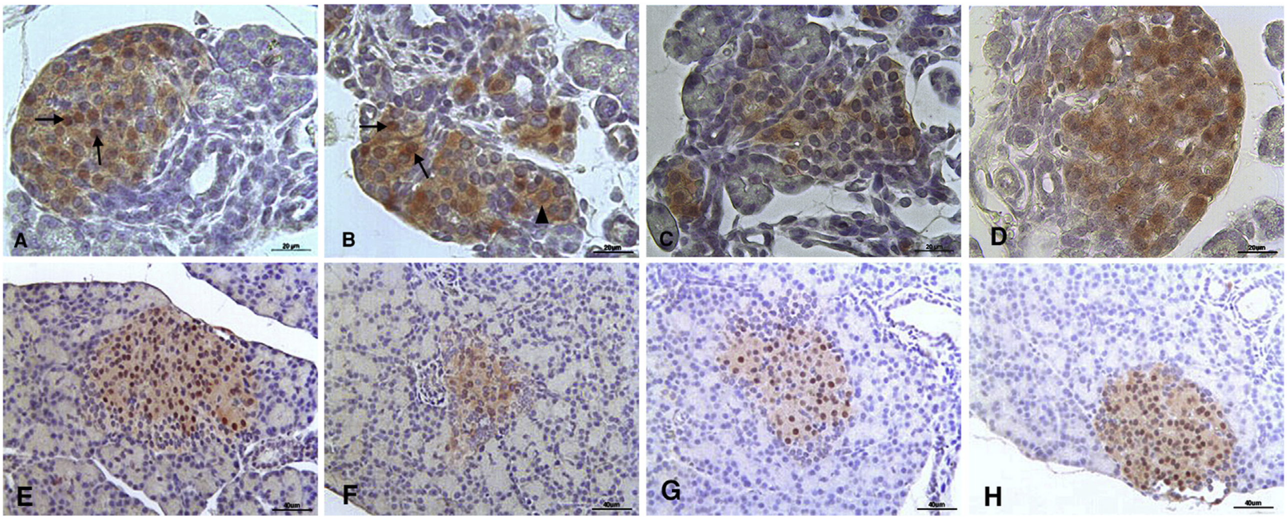


Fig. 3. Immunolocalization of Pdx-1 in the pancreas sections of the short-term (A–D) and the long-term (E–H) groups. Pdx-1 nuclear positive (N+) (↑), cytoplasmic positive (C+) (▲) cells. A,E; Control, B,F; n2-STZ, C,G; n2-STZ+VG, D,H; VG groups. Immunostaining: Streptavidin-biotin-peroxidase, Counterstaining: Hematoxylin, Magnification: A–D: Bar, 40 µm; E-F: Bar, 20 µm.

Table 4
Comparison of Pdx-1 positive cell numbers in small islets, exocrine tissue and pancreatic ducts in all groups of short-term (A) and long-term (B) in newborn rats.

	Number of Pdx-1 (+) cells in islets (%)			
	Control	n2-STZ	n2-STZ + VG	VG
		Short-term		
Nuclear Pdx-1(+)	70.372 ± 1.69	40.97 ± 3.21^{a}	60.052 ± 4.09^{b}	68.830 ± 2.59^{e}
Cytoplasmic pdx-1(+)	73.597 ± 2.20	43.58 ± 3.59^{c}	65.818 ± 3.47^{d}	73.375 ± 2.51^{f}
		Long-term		
Nuclear Pdx-1(+)	73.27 ± 2.76	43.228 ± 3.37^{a}	60.602 ± 4.82^{b}	74.550 ± 3.12
Cytoplasmic pdx-1(+)	82.54 ± 4.55	48.923 ± 4.06^{c}	66.662 ± 2.44^{d}	74.503 ± 2.52^{e}

$^{a,c}p < 0.001$ *versus* control; $^{b,d}p < 0.001$, *versus* n2-STZ; $^{e,f}p < 0.001$, *versus* n2-STZ + VG (Short-term).
$^{a,c}p < 0.001$ *versus* control; $^{b,d}p < 0.001$, *versus* control; $^{e}p < 0.01$ *versus* control (Long-term).

number of intra-islet apoptotic cells increased significantly in the n2-STZ group, compared to the other groups ($p < 0.001$). Apoptotic cell numbers in the n2-STZ + VG group decreased significantly, compared to the n2-STZ group ($p < 0.001$). A few individual TUNEL-positive cells were detected in exocrine tissue and within the duct epithelial cells of all groups (Fig. 4, Table 6), No TUNEL-positive cells were detected in the islets of the long-term subgroups, whereas a few TUNEL-positive cells were found in the exocrine tissue and duct epithelium (data not shown).

DDR-1

As a result of immunohistochemical staining for DDR-1 in pancreatic tissue sections, cells with stained plasma membranes were evaluated as DDR-1 plasma membrane positive (PM+) cells. Additionally, there were specific cytoplasmic (C+) or cytoplasm/plasma membrane (C+/PM+) immunopositive cells, which are common particularly in intra-islet endocrine cells. DDR-1 positive cells in islets and exocrine tissue were counted in the short- and long-term groups, considering all the abovegiven immunostaining characteristics (PM+, C+, C+/PM+), and statistically evaluated (Fig. 5; Table7). DDR-1 C+/PM + cells generally located at the center of the islets. The number of DDR-1 PM + cells in the control group was lower than all the other groups, and these positive cells located close to the center. PM + cell numbers increased significantly in the n2-STZ diabetic group compared to the control ($p < 0.001$), similarly in the n2-STZ + VG group. Moreover, their localization was from the center to the periphery. PM + cells in the VG group also showed dispersed localization, and the number of positive cells increased compared to the control group ($p < 0.001$). For all short-term groups, the number of DDR-1 plasma membrane positive cells in small 5–10 cell islets increased in the duct periphery and in the exocrine tissue of the n2-STZ + VG group ($p < 0.001$). All data is shown in Fig. 5 and Table 7.

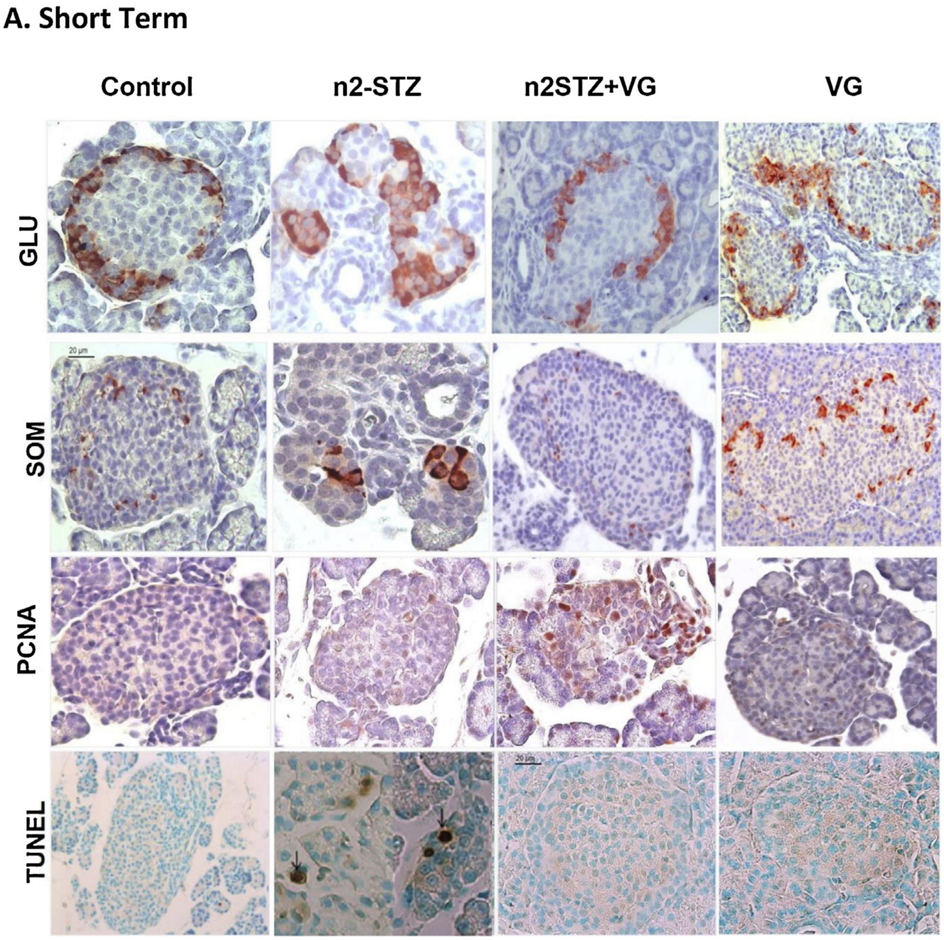
DDR-1/insulin double staining

Immunopositive cells for both insulin (cytoplasmic-brown colored) and DDR-1 (membrane-red colored) were evaluated by double staining to demonstrate localization of insulin and DDR-1 proteins at the same cell, (Fig. 6). Interestingly, the short-term groups showed more distinctive double immunopositive cells. The number of double immunopositive β cells for insulin and DDR-1 was significantly higher in the n2-STZ + VG group compared to the other groups ($p < 0.001$) (Fig. 7). Reduced numbers and weakly stained double immunopositive cells were found in the long-term groups but not evaluated.

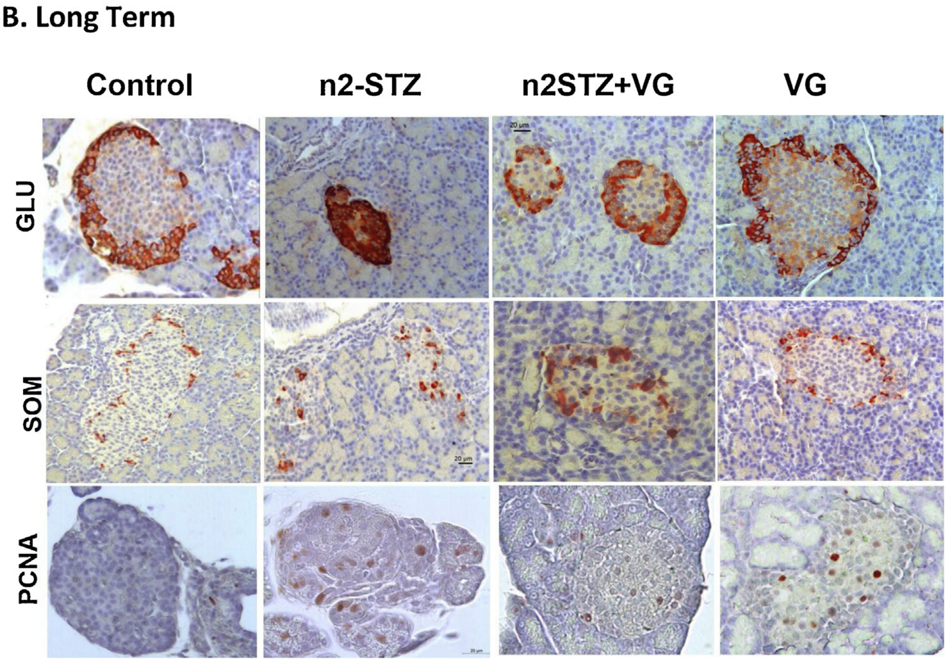
Discussion

The present study revealed the effects of short and long-term VG treatment on the mechanisms contributing to β cell mass and regeneration in the n2-STZ diabetic model, as well as determined the endocrine progenitor cells expressing DDR-1 which have not been studied to date. STZ-induced neonatal diabetic rats are a useful experimental model for the regeneration of β cells [17]. Rapid regeneration was detected as newly formed β cells and clusters seen in intra-islet and extra-islet tissue, such as exocrine tissue and duct epithelium [15,18,19]. During the rapid pancreatic growth and the formation of new β cell mass in neonatal rats on the first day of birth, high levels of β cell apoptosis and proliferation have been observed [20]. However, following the first week of birth, β cell apoptosis was revealed to be at the highest level while replication of β cells was at the lowest level, compared to the other weeks. This study showedthat VG treatment induced β cell replication by 8.5-fold, but decreased β cell apoptosis during the newborns' first week, and caused an increase in the total number of immature pancreatic β cells and newly differentiated β cells [21]. VG inhibits the DPP4 enzyme, participating in the regeneration of cells by increasing endogenous GLP-1 levels, which is known to decline in Type 2 diabetic patients. VG has been reported to participate in growing β cell mass *via* several molecular mechanisms. Studies have disclosed that VG induces DNA replication in β cells and activates adenylyl cyclase and cAMP/PKA signal pathways, in addition to other MAPK, epidermal growth factor, PKAB, and PKC pathways [22,23]. This study revealed that the β cell mass and size of islets, including the insulin-positive cells, significantly increased in the VG treatment only groups in both the short and long-term, in comparison with the controls. We also observed a notably significant increase in PCNA-positive cells in both the VG treated diabetics and VG groups. There was not any significant difference in the apoptotic index between the short-term control and VG applied only groups. An apparent loss of β cells was observed in n2-STZ diabetics, whereas VG markedly inhibited apoptosis in VG-applied diabetics. We detected new β cell masses generated in both intra-islet and extra-islet exocrine pancreas tissue, as well as in the ducts of VG-treated diabetic groups, suggesting that both short-term and long-term VG application in STZ diabetics may have significant roles in the survival of β cells *via* inhibiting apoptosis and protecting GLP-1 levels. Also, VG treatment may affect the mechanisms for β cell mass increase by replication of present β cells or by neogenesis of progenitor cells, as reported in previous studies [15,24,25].

VG inhibits the degradation of GLP-1, a cell-growth and survival factor, and increases plasma glucose-dependent insulin secretion, leading to an increase in plasma GLP-1 levels, and significantly regulates plasma glucose levels. GLP-1 plays a crucial role in the growth and differentiation of pancreatic β cells [23,26], and protects β cells from apoptotic cell death [27]. However, GLP-1 is quickly broken down by a DPP4 enzyme in diabetics, [28]. It has been reported that the using of a DPP4 inhibitor increases the levels of circulating endogenous GLP-1, and also to ameliorate the glycemic index in both rodents and Type 2 diabetic patients [29]. In our study, short-term and long-term VG application mediated the reduction of blood glucose levels through the insulin-regulating effect of GLP-1 in VG-treated diabetics, compared to STZ diabetics;



A. Short Term



B. Long Term

Fig. 4. Immunolocalization of glucagon, somatostatin, PCNA and TUNEL in the pancreas of the short-term (A) and the long-term (B) groups. Immunostaining: Streptavidin-biotin-peroxidase and TUNEL method. Counterstaining: Hematoxylin for IHC, methyl green for TUNEL. Magnifications: 200x.

Table 5
Comparison of areas covered by glucagon and number of somatostatin cells (%) in islets.

	Control	n2-STZ	n2-STZ + VG	VG
	Areas covered by Glucagon (+) cells in islets (%)			
Short Term	30.355 ± 1.93	53.61 ± 2.97^{a}	36.612 ± 0.66^{b}	26.68 ± 0.88^{c}
Long Term	27.663 ± 4.16	52.35 ± 10.48^{d}	28.375 ± 3.79^{e}	12.96 ± 0.42^{f}
	Number of somatostatin cells in islets			
Short Term	7.925 ± 0.07	21.143 ± 0.98^{a}	9.505 ± 0.22^{b}	2.947 ± 0.29^{g}
Long Term	8.007 ± 0.52	20.402 ± 1.74^{d}	8.125 ± 0.99^{e}	13.583 ± 1.69^{h}

$^{a,d}p < 0.001$ *versus* control; $^{b,e}p < 0.001$, *versus* n2-STZ; $^{c}p < 0.05$ *versus* control; $^{f}p<0.01$, *versus* control, $^{g,h}p < 0.001$ *versus* control.

Table 6
PCNA and TUNEL positive cell number in short and long term.

	Control	n2-STZ	n2-STZ + VG	VG
	Number of PCNA cells in islets			
Short Term	0.1 ± 0.01	2.71 ± 0.14^{a}	6.24 ± 0.1^{b}	4.01 ± 0.07^{c}
Long Term	0.28 ± 0.01	2.01 ± 0.004^{d}	4.01 ± 0.002^{e}	7.23 ± 0.45^{f}
	Apoptotic cell number in islets			
Short Term	0.023 ± 0.01	5.75 ± 1.31^{c}	0.28 ± 0.01	0.023 ± 0.017

$^{a,d}p < 0.001$ *versus* control; $^{b,e}p < 0.001$, *versus* n2-STZ; $^{c}p < 0.05$ *versus* control; $^{f}p < 0.01$, *versus* control for PCNA. $^{c}p < 0.001$ *versus* control, n2-STZ + VG and VG for TUNEL

these effects were also reported in a previous study [21]. There is an association between DPP4 inhibitors and weight loss or weight neutrality in Type 2 diabetics [30]. Therefore, it is reasonable to observe a reduction in the body weights of VG-treated n2-STZ diabetics at the end of a ten-day treatment regimen in this present study. A decrease in the VG treated diabetic group's blood glucose levels was also observed in comparison to the STZ diabetic group in the long-term application, whereas the BWs did not change. Glycemic control regulated by an agent such as VG, which increases insulin production or enhances insulin function, can result in weight gain. On the other hand, hypoglycemic episodes can also promote weight gain. The islet sizes and the beta cell numbers increased in only the VG treated group at long-term application, suggesting that a lower blood glucose level in the VG group due to the increase in insulin production and release may cause defensive eating to prevent a decline from normoglycemia to hypoglycemia, and therefore cause weight gain. The mechanisms underlying the body weight gain or loss secondary to VG treatment are not apparent yet. However, it can be concluded that the effects of VG on fat metabolism could be associated with body weight regulation. All potent mechanisms should be examined by further studies covering the longer-term treatment by VG. Cellular and molecular mechanisms of DPP4 inhibitors mediating the β cell increase have not been fully understood yet. In terms of preventing diabetic progression, the therapeutic role of DPP4 inhibitors can be explained primarily by the mechanisms of β cell regulation in neonatal rats.

Many transcription and growth factors affect the formation and differentiation of both the exocrine and endocrine pancreas [31]. Pdx-1, a transcription factor, essentially plays a role in pancreatic embryonic development and β cell differentiation [32]. Pdx-1 expression in adults is limited to β cells and a small number of somatostatin cells specifically [33]. Guz et al [34] have shown that some of the islet cells can transform into β-cells. Kodama et al [35] reported co-localization of Pdx-1 (+) cells with somatostatin (+) cells, which control insulin gene expression in adult β cells. Claims suggest Pdx-1 expressing duct cells are progenitor cells which transiently regain their pluripotent characteristics in the adult pancreas [15,24]. In the present study, we observed that the number of Pdx-1 (+) cells decreased in the pancreatic tissues of STZ diabetic rats while increasing in VG treated diabetics. So, we suggest that Pdx-1 positive cells in the exocrine tissue and pancreatic ducts might be beta precursor cells, supported by a positive effect of VG on islet cell regeneration.

Cellular localization of Pdx-1 has been shown to vary according to various metabolic conditions. Intra-nuclear or extra-nuclear localization of Pdx-1 is significant in regulating Pdx-1 functions. In the case of low glucose levels, Pdx-1 localizes at the nucleus' periphery adjacent to the cytoplasm of islet cells, whereas at high glucose levels, it is transferred from the nucleus' periphery to the nucleoplasm [36,37]. Pdx-1 is translocated to the cytoplasm during oxidative stress based on diabetic state [38,39]. Cytoplasmic Pdx-1 has been claimed to contribute to a specific cytoplasmic function in some pathological conditions [40]. In this study, we observed nuclear localization, as well as cytoplasmic localization of Pdx-1 in n2-STZ diabetic groups, suggesting that it may be dependent on ER stress or similarly unfavorable conditions resulting from Type 2

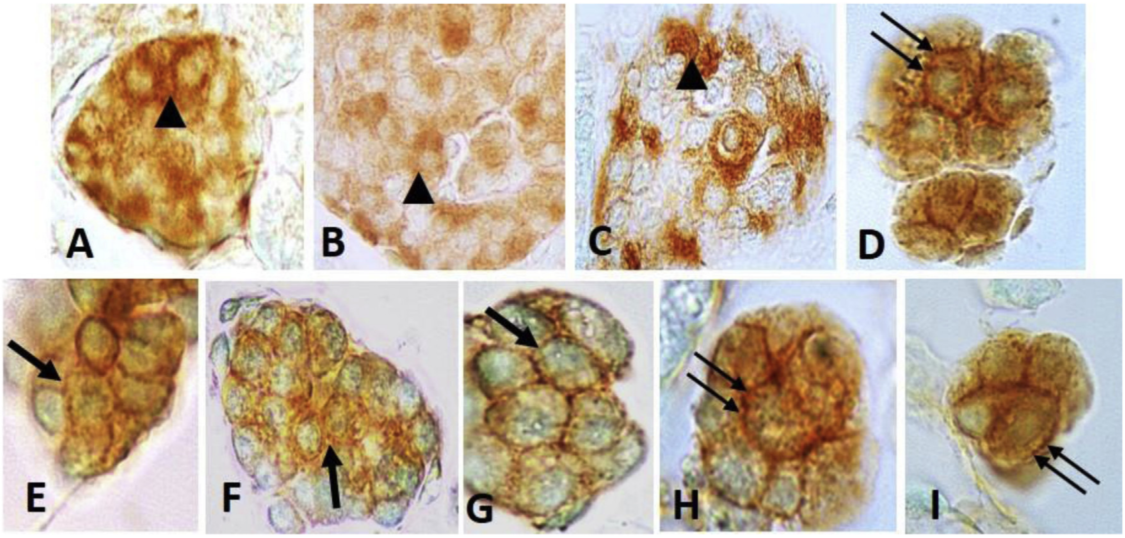


Fig. 5. Immunolocalization of DDR-1 in the pancreas sections of the short-term groups. (A,B) Control group; (C–D) n2-STZ group; (E–G) n2-STZ + VG group; (H, I) VG group. DDR-1 plasma membrane positive (PM+) (↑), cytoplasmic (C+) (▲), cytoplasm + plasma membrane (C+/PM+) (↑↑) cells. Immunostaining: Streptavidin-biotin-peroxidase, Counterstaining: Methyl green. Magnification: A–C: 200x ; F: 400x ; D, E, G-I: 1000x.

Table 7
DDR-1 immunopositive cell number (%) in short- and long-term.

DDR-1 immunopositive cell number (%)					
DDR1		Control	n2-STZ	n2-STZ + VG	VG
			Short Term		
Intra islets	**PM+**	3.410 ± 0.41	7.927 ± 1.19^{a}	11.462 ± 1.19^{b}	10.397 ± 0.84^{c}
	C+/PM+	20.345 ± 0.74	14.752 ± 0.88^{d}	16.648 ± 0.82^{e}	32.343 ± 0.86^{f}
	C+	96.110 ± 2.45	61.525 ± 3.78^{r}	92.395 ± 1.37^{s}	95.850 ± 1.86
	PN+	12.467 ± 0.97	9.337 ± 0.92^{t}	14.797 ± 0.77^{u}	16.698 ± 1.26^{r}
Extra- islets	**Exocrine PM+**	1.833 ± 0.75	6.00 ± 0.89^{h}	8.167 ± 0.75^{g}	3.500 ± 0.54^{i}
	Small islets PM+	0.500 ± 0.54	3.66 ± 0.51^{j}	7.167 ± 0.75^{k}	1.667 ± 0.51^{m}
	Cell clusters PM+	0.510 ± 0.55	3.64 ± 0.50^{n}	$10.667 \pm 0.51^{\circ}$	7.333 ± 0.51^{p}
			Long Term		
Intra- islets	**PM+**	1.320 ± 0.36	0.958 ± 0.055	4.922 ± 0.82^{a}	9.840 ± 1.08^{b}
	C+/PM+	4.265 ± 0.27	2.905 ± 0.108	$71.520 \pm 3.49^{c, d}$	18.363 ± 0.98^{e}
	C+	86.385 ± 0.89	93.135 ± 1.080^{f}	72.343 ± 3.085^{g}	77.835 ± 1.79^{h}
	PN+	3.508 ± 0.35	2.057 ± 0.038^{j}	6.170 ± 0.36^{k}	9.072 ± 0.90^{l}
Extra- islets	**Exocrine PM+**	0.505 ± 0.54	1.667 ± 0.516^{m}	9.667 ± 0.816^{n}	$8.500 \pm 0.54^{\circ}$
	Small islets PM+	0.010 ± 9.27	$0.340 \pm 0.511^{*}$	4.500 ± 0.54^{p}	5.167 ± 0.75^{r}
	Cell clusters PM+	0.835 ± 0.40	1.667 ± 0.516	3.500 ± 0.54^{s}	5.000 ± 0.63^{t}

^{a,c,f,d,h,j,n,r,t,p} $p < 0.001$, $^{i}p < 0.01$, $^{m}p < 0.05$ *versus* control group ^{b,s,u,g,k,o} $p < 0.001$, $^{e}p < 0.01$ *versus* n2-STZ group for short-term. $^{f,\ j,c,*}p < 0.001$ *versus* control group; ^{a,d,g,k,n,p,} $^{s}p < 0.001$ *versus* n2-STZ group; $^{b,e,h,l,o,r,t}p < 0.001$, $^{m}p < 0.05$ *versus* control group for long-term. DDR-1 plasma membrane positive (PM+), DDR1 cytoplasmic/plasma membrane immunopositive (C+/PM+), DDR1 cytoplasmic immunopositive (C+) and DDR1 perinuclear positive (PN+) cell. (Cell cluster: 2–3 cells; small islet: 5–10 cells).

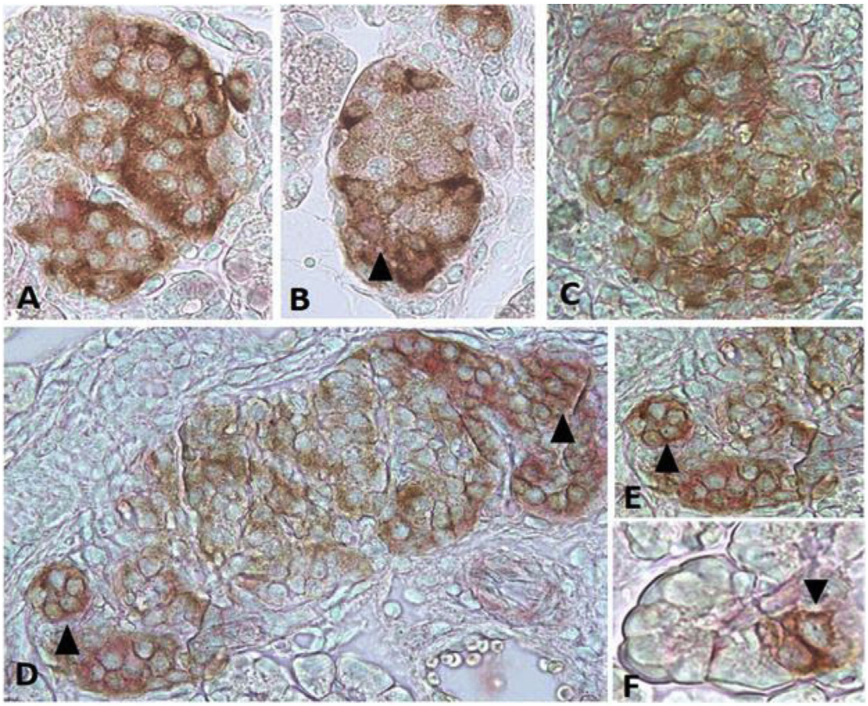


Fig. 6. Double- immunolocalization of DDR-1 and insulin (▲) in the short-term group. (A) Control group; (B) n2-STZ group; (C–F) n2-STZ + VG group. Double-Immunostaining, Counterstaining: Methyl green. Magnification: A-E: 400x ; F: 1000x.

diabetes or related to different physiological conditions. To explain these relationships, more advanced studies are needed.

Glucagon and somatostatin cells are usually localized at the intra-islet periphery. In a previous study, we reported disorganization of these intra-islet cells as well as an increase in the number of these cells in the n2-STZ diabetes models [15]. In this study, we reported that the impaired morphology of islets due to STZ application returned to its normal state after VG application, presenting an effective role of VG on islet morphology.

There are several effective approaches to preventing loss of β cells or replacing the cells as treatment of Type 2 diabetes. Mostly, β cells have been implemented in replication, neogenesis or transdifferentiation by inducing regeneration of endogenous β cells [41,42]. Several biomarkers have been reported to detect

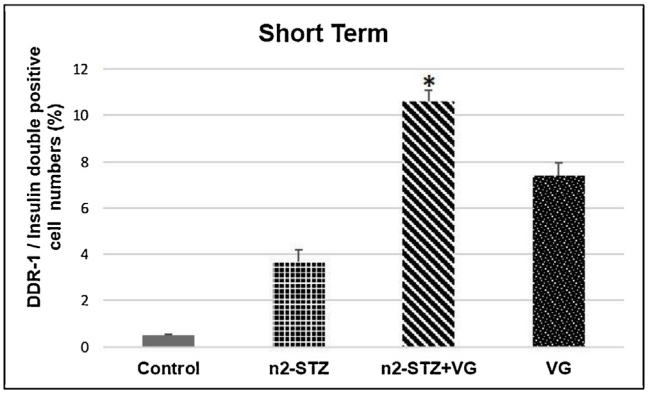


Fig. 7. DDR-1 / Insulin double positive cell numbers of short-term groups. $^{*}p < 0.001$ *versus* the other groups.

and choose specific intra-islet and/or extra-islet endocrine progenitors. Hald et al. [12] performed extensive scanning for endocrine progenitor cell markers to identify cell surface markers of progenitor pancreatic islet cells existing during both embryonic and adult periods. They examined the co-localization of transcription factors and biomarker candidates in β cell development and found that one of the candidate molecules, DDR-1, co-localized with Ngn3 (+) cells and NKX6.1 (+) cells, progenitors of the islet endocrine cells. DDR-1 expression was detected in the embryonic period, while not detected in adult human and mouse pancreases [12,43], and is accepted as a multipotent pancreatic progenitor marker. Xu et al. [2] performed a pancreatic duct ligation (PDL) and detected Ngn3 (+) cells in both intra-islet and extra-islet exocrine tissue and ductal epithelium allowing for an increase in Ngn3 expression, which is a progenitor cell marker in islets with a parallel increase in β cell number. Supportive data about this issue came from the studies of Hald et al. [12], reporting re-expression of DDR-1 by PDL in 8 week-old balb/c mice. They also reported that DDR-1 expression had high positivity in Pdx-1 positive progenitor regions.

There are no studies about the distribution of DDR-1 expression in the endocrine and exocrine pancreas under diabetic conditions. This study is the first report investigating the induction of DDR-1 expression by the DPP4 inhibitor VG in potential islet progenitor cells, identifying a relation to β cell regeneration in the n2-STZ diabetic model.

Despite well-known functions during the embryonic period, the roles of DDR-1 in adults have not been identified yet. There is contradictory data on the pancreatic expression of DDR-1. Some researchers have asserted that DDR-1 expression was limited to endocrine cells in an adult pancreas [44], while Hald et al. [12] claimed that this expression only occurs in the embryonic period, not in the adult pancreas. In this study, we evaluated the distribution and numbers of DDR-1 expressed cells, and classified them according to their immune reaction localization in the pancreatic tissue sections. Since DDR-1 essentially functions in the cell membrane, a principal evaluation was performed for the plasma membrane positivity (PM+). Moreover, the cytoplasmic DDR-1 positive cells were also evaluated since they are located in the islets specifically. Considering all types of the immunostaining, we found DDR-1 expression in the healthy adult pancreas, even in scant amounts, and that VG application induced a marked increase of DDR-1expression.

In this study, we observed that VG application in both the short and long-term groups caused an increase in the number of intra-islet individual DDR-1 (+) cells or extra-islet cell clusters. STZ application also induced DDR-1 expression in both of the groups. On the other hand, it has been reported that increased DDR-1 expression at genotoxic conditions depends on the levels of p53 or XRCC3 as DNA repair proteins [45,46]. Thus, we suggest that DDR-1 expression may be induced by STZ genotoxicity in STZ applied rats.

There was a marked increase in the number of plasma membrane DDR-1 (+) cells in VG applied groups (n2-STZ + VG and VG), compared to the n2-STZ and control groups. Intra-islet DDR-1 (M+) cells are generally distributed from the center to the periphery of the islets; this distribution was similar to β cells within the islets. Furthermore, insulin and DDR-1 positive cells detected by double immunostaining may be considered as progenitor cells. According to our results, we suggest that VG induces and increases DDR-1 expression, allowing induction of progenitor β cells, contributing to the mechanisms of differentiation into β cells. We also identified a decrease in perinuclear DDR-1 positivity (DDR-1 PN+) in the short and long-term diabetic groups, but VG application in STZ diabetics and non-diabetics caused a marked increase in PN + DDR-1 cells. The perinuclear localization of DDR-1 has not been shown until now, this study is the first report in this issue. There are 5-isoforms of DDR-1 (DDR-1a, DDR-1b, DDR-1c, DDR-1d, and DDR-1e) emerged by alternative splicing [13]. We suggested that the perinuclear positivity of DDR-1 can be related to an alternative function of some of these isoforms in the nucleus. The cytoplasmic DDR-1 positivity identified in this study may be possibly related to the presence of DDR-1 in the secretory vesicles stored in the cytoplasm until they are released to the plasma membrane in case of an active protein.

The Ras / Raf / ERK signal pathway regulates DDR-1 transcription in smooth muscle, mammary epithelium, megakaryocytes and embryonic renal cells [47]. It has been shown that collagen-1 induces DDR-1 expression depending on ERK 1/2 in lung fibroblasts [48]. Under genotoxic stress conditions, DDR-1 activates the NF-kB pathway in MCF7 mammary cancer cells, and the survival signals are induced by DDR-1 *via* Notch signaling in HCT116 colon carcinoma cells. DDR-1 in these cells was also shown to induce its own expression through the Ras / Raf / ERK signal pathway [45]. In many cases, the pathways regulating DDR-1 transcription have not been clearly understood yet. Increased DDR-1 expression in our VG-applied diabetic group may be related to activation of MAPK by VG and modulation of DDR-1 transcription *via* the Ras / Raf / ERK signal pathway. It is possible that VG effected cell replication mechanisms *via* GLP-1, a cell growth and survival factor, while it might also induce the transcriptional mechanisms of DDR-1 by using a similar function. As stated above, the survival signals induced by DDR-1 are collected *via* Notch signaling [49]. Due to the protective effect of VG against the apoptotic cell death of β cells, VG application in neonatal rats may activate survival signals by the Notch pathway and cause an increase of DDR-1 expression. The increase of β cell mass by VG application may be due to replication of existing β cells, neogenesis of progenitor cells, or transdifferentiation of some other differentiated mature cells into β cells. Our findings supported that VG induces and/or increases the expression of DDR-1, participating in the increase of β cell mass through neogenesis of progenitor cells.

Taken together, VG promotes β cell neogenesis in the duct epithelium and acinar cells, stimulates the transformation of the DDR-1 expressing endocrine progenitor cells into β cells. VG also induces the proliferation of islet cells by increasing PCNA expression, reduces islet cell apoptosis, and regulates the morphological reorganization of islets in STZ diabetic neonatal rats.

In this study, we showed that DDR-1, which was previously reported to be expressed only in the embryonic period, was stimulated in the postnatal period by DPP4 inhibitor administration. Further studies will reveal how the progenitor cells expressing DDR1 may have been transformed into other adult endocrine cells other than beta cells and which mechanisms are involved in this process. Our study is the first report mapping immunolocalization of DDR-1 in the pancreas by short-term and long-term application of VG both in diabetic and non-diabetic neonatal rats. We suggest that our results may contribute to the development of a new therapeutic model for the treatment of diabetes by an approach based oninduction of pancreatic β cell regeneration and differentiation of progenitor endocrine cells into β cells.

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