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Immunohistochemical, apoptotic and biochemical changes by dipeptidyl peptidase-4 inhibitor-sitagliptin in type-2 diabetic rats

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ABSTRACT

Background: Diabetes is a major public health problem that is rapidly increasing in prevalence. In this study, the effects of sitagliptin, a dipeptidyl peptidase-4 inhibitor, were examined on newborn diabetic rat model.
Methods: Wistar albino newborn rats were divided into control (Ctrl), sitagliptin (Sit), diabetic and diabetic + Sit groups. On the second day after the birth, 100 mg/kg streptozotocin (STZ) was administered intraperitoneally in a single dose to induce type-2 diabetes in rats. The Sit and diabetic + Sit groups were administered sitagliptin (1.5 mg/kg subcutaneous) as of the day 5 for 15 days. The pancreas sections were stained with insulin (INS), glucagon (GLU), somatostatin (SS), glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-1 receptor (GLP-1R) antibodies by the streptavidin–biotin peroxidase technique. The TUNEL method for apoptosis and biochemical analysis were performed in the pancreas and serum, respectively.
Results: Body weight and blood glucose levels showed significant differences among all groups on days 11 and 20. In diabetic rats following treatment with sitagliptin, the area percentage of INS immunopositive cells increased while the area percentage of SS immunopositive cells decreased, insignificantly. A significant increase was observed on the area percentage of GLU, GLP-1 and GLP-1R immunopositive cells in the diabetic + Sit group when compared to the diabetic group. The area percentage of apoptotic cells was the same among all groups. While serum glutathione and malondialdehyde levels demonstrated insignificant alterations, the catalase and superoxide dismutase activity significantly changed among four groups.
Conclusion: According to our findings, sitagliptin may be a useful therapeutic agent to a certain extent of type-2 diabetic condition.
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Introduction

Diabetes mellitus is one of the most common metabolic diseases in the world that is characterized by increased blood glucose level during fasting or oral glucose tolerance test [1,2]. Type-2 diabetes is identified by means of impairments in the β-cell function and insulin (INS) secretion [1,3]. Streptozotocin (STZ) is widely preferred in investigations as part of studies of the type-2 diabetic model [4]. In animal models, STZ is administered to newborn rats to induce type-2 diabetes [5].

Incretin hormones are important to understand the mechanism of glucose homeostasis. Glucagon-like peptide-1 (GLP-1), one of the most important of incretin hormones, increases insulin secretion after food ingestion [6]. It is suggested that GLP-1 receptor (GLP-1R) is expressed on α and β cells of pancreas, gastrointestinal system, kidney, lung, heart and brain [7,8]. It has been reported that GLP-1 provides β cell regeneration, differentiation and prevents apoptosis *via* the activation of GLP-1R [9,10]. GLP-1 hormones are degraded by the dipeptidyl peptidase-4 (DPP-4) enzyme [11]. DPP-4 is expressed in pancreas, brain, lung, kidney, liver, intestine, adrenal gland and lymphocytes [12]. Long-term studies suggest that DPP-4 inhibitors decreased β cell death, increase its neogenesis and function in diabetic animals [13,14].

Sitagliptin, a novel DPP-4 inhibitor, is a therapeutic agent for type-2 diabetes that is administered either as a monotherapy or

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combination therapy [15,16]. Furthermore, the agent provides improvements in glycemic control [17]. DPP-4 inhibitors-related studies on humans have found a decrease in HbA1c levels [18]. It has been reported that DPP-4 inhibitors do not affect body weight [19]. Moreover, it is suggested that sitagliptin may have a role as a treatment agent for pre-diabetes [20].

In our study, we aimed to explore the biochemical alterations in serum, INS, glucagon (GLU), somatostatin (SS), GLP-1 and GLP-1R expressions and programmed cell death (apoptosis) in the pancreas of healthy and type-2 diabetic rats following sitagliptin administration.

Materials and methods

Animals and tissue preparation

All animal experimental procedures were approved by the Istanbul University Local Ethics Committee on Animal Research. When the newborn Wistar albino rats were 48-h-old following birth, they were included in the experimental groups. There were four groups of newborn rats. Group I ($n = 8$): the control group (Ctrl) consisted of newborn rats that were given physiological saline for a period of 18 days, intraperitoneally (*ip*). Group II ($n = 8$): the sitagliptin group (Sit) was treated with 1.5 mg/kg/day sitagliptin (Januvia, Merck) dissolved in physiological saline from the fifth day for a period of 15 days, subcutaneously (*sc*). Group III ($n = 8$): for streptozotocin (STZ)-induced diabetic rats (diabetic), 100 mg/kg STZ (Sigma–Aldrich, S0130) was dissolved in physiological saline and given as a single intraperitoneal dose on the second day following birth [21,22]. The rats whose blood glucose levels were 200 mg/dl or more on the second day following STZ injection were considered as diabetic. Group IV ($n = 8$): the diabetic group which was given sitagliptin (diabetic + Sit) on the fifth day following birth for a period of 15 days.

The blood glucose levels of animals in all groups were measured with a glucometer (Accu-check, Roche Diagnostics GmbH) using the blood samples collected from the tail vein on days 2, 4, 11 and 20. The body weights of the animals were also measured at the same time intervals.

On the 20th day, the animals were left to fast for 1 h; blood and pancreatic tissue samples were collected under ketamin-HCl (50 mg/kg) anesthesia. The tissue samples were fixed in 10% neutral buffered formalin for 24 h at +4 °C and then embedded in paraffin using routine light microscopy processing methods.

The study of Langerhans islet size and immunohistochemical staining

The sections of pancreases were stained by using hematoxylin and eosin. All of the Langerhans islets in pancreas were classified as small, medium and large according to their size after calculating the areas. Large islets were >10,000 m^2, medium islets were >5000–10,000 m^2, and small islets were <5000 μm^2 [23].

For immunohistochemical staining, the sections were incubated with mouse monoclonal INS (dilution 1:1000; Sigma–Aldrich, I2018), mouse monoclonal GLU (dilution 1:2000; Sigma–Aldrich, G2654) and rabbit polyclonal SS (dilution 1:2500; Chemicon, AB1976), mouse monoclonal GLP-1 (dilution 1:500; Santa Cruz, sc57166) and rabbit polyclonal GLP-1R (dilution 1:50; Novus, NLS1205) antibodies. They were stained with the streptavidin–biotin-peroxidase method using histostain-plus bulk kit (Zymed 85-9043). The detection procedures were carried out as described by the manufacturer. The enzyme activity was developed using 3-amino-9-ethyl-carbazole (AEC) substrate kit (Zymed 00-2007) and the sections were then counterstained with Mayer's hematoxylin. The staining intensity was scored from one to three as weak (+), medium (++) and strong (+++). The negative control sections were prepared by means of substituting antibodies with phosphate buffer saline (PBS). The rat stomach sections were used as SS positive controls and non-experimental rat pancreas sections were used as INS, GLU, GLP-1 and GLP-1R positive controls because of their high expressions.

The photographs of the Langerhans islets in pancreas were taken and the size of islets calculated using a Nikon Eclipse 80i light microscope equipped with a digital camera using NIS-Elements-D 3.1 microscope imaging software program.

Apoptosis

The apoptosis was determined by the terminal deoxynucleotidyl transferase (Tdt) mediated dUTP (TUNEL) method (APOP-TAG® kit; Millipore S7101) according to the manufacturer's instructions. The reaction was observed using an AEC substrate kit. The negative control sections were prepared by substituting Tdt enzyme with distilled water. The rat breast tissue sections (S7115) were used as positive controls for the detection of programmed cell death.

Biochemical assay

The blood samples were then centrifuged at 3000 rpm for 10 min to separate the serum. The biochemical parameters were determined on serum samples were frozen at −80 °C. Serum glutathione (GSH), malondialdehyde (MDA) levels, catalase (CAT) and superoxide dismutase (SOD) activities were measured. GSH levels were determined according to Beutler's method using metaphosphoric acid for protein precipitation and 5,5′-dithiobis (2-nitro benzoic acid (Sigma–Aldrich) for the color development at 412 nm [24]. The MDA levels were estimated by using the Ledwozyw's methods [25]. The serum samples were mixed thoroughly with a solution of trichloroacetic acid (TCA) (Sigma–Aldrich) (30%), TBA (Merck) (0.75%) and 5 M hydrochloric acid. The samples were measured at 535 nm. The CAT activity was assayed by the methods of Aebi. The reaction mixture was made up of the sample, 50 mM phosphate buffer pH 7.0 and 10 mM H_2O_2. The reduction rate of H_2O_2 was observed to be 240 nm at the room temperature for 60 s [26]. The SOD activity was determined by the method developed by Beauchamp and Fridovich that involves the inhibition of nitroblue tetrazolium (NBT) (Sigma–Aldrich, Louis) [27]. The protein content in the serum was estimated by the method of Lowry [28].

Statistical analysis

The INS, GLU, SS, GLP-1, GLP-1R immunopositive cells and apoptotic cells were counted in all islets of Langerhans for each section of rat pancreas. The area percentages of immunopositive and apoptotic cells in the islets were calculated by using the formula of (labeling area/total area) × 100. The values obtained were evaluated statistically.

SPSS 21.0 software was used for the statistical analysis. The results were expressed as means ± SEM. The statistical evaluations were performed using Kruskal–Wallis test followed by Mann–Whitney *U* test. A *p*-value <0.05 was considered to be significant.

Results

Body weight and blood glucose

In Figs. 1 and 2, the changes in the comparable body weight and blood glucose levels for all experimental groups are presented. A significant difference in body weight and blood glucose level was

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