International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2024.1292.1300

Research Article
Leptin and Insulin Interaction Modulates Pancreatic Nitric Oxide Levels in Diabetic Rats

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Abstract

Background and Objective: Diabetes mellitus, characterized by hyperglycemia due to insulin secretion deficits, involves the crucial influence of leptin on pancreatic hormones. Nitric oxide (NO), a key signal molecule, is vital for leptin's action in various cell types, including pancreatic β-cells. This study investigates the interplay of leptin and insulin with NO in streptozotocin-induced diabetic rats. **Materials and Methods:** Rats were categorized into seven groups: Control, Streptozotocin (Diabetes), Leptin, Insulin, Streptozotocin+Leptin, Streptozotocin+Insulin and Streptozotocin+Leptin+Insulin. Regular blood glucose measurements, serum leptin and insulin level determinations, immunohistochemical detection of nitric oxide synthases and leptin expressions and histopathological examinations were conducted. **Results:** Blood glucose levels in Streptozotocin+Insulin and Streptozotocin+Leptin+Insulin groups closely resembled those in the control group. Weaker inducible nitric oxide synthase immunoreactivity was observed in the Streptozotocin+Leptin, Streptozotocin+Insulin and Streptozotocin+Leptin+Insulin groups compared to the Streptozotocin group. Positive leptin immunoreactivity was intense in the islets of the Streptozotocin+Leptin+Insulin group. **Conclusion:** Combined leptin and insulin treatment normalized hyperglycemia in diabetic rats by suppressing inducible nitric oxide synthase availability and enhancing insulin sensitivity in the pancreas.

Key words: Diabetes, insulin, leptin, nitric oxide synthase, pancreatic tissue, streptozotocin-induced rats

Citation: Yılmazer, N., A. Kapucu, E.G. Gürevin, S. Üstünova and A. Kandil *et al*., 2024. Leptin and insulin interaction modulates pancreatic nitric oxide levels in diabetic rats. Int. J. Pharmacol., 20: 1292-1300.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus (DM) is a global health concern encompassing a group of metabolic diseases, primarily characterized by hyperglycemia stemming from defects in insulin secretion and/or action and disrupted carbohydrate, fat and protein metabolism[1,2]. Pancreatic β-cell dysfunction plays a pivotal role in the pathogenesis of both type 1 and 2 diabetes[3], as these cells are responsible for synthesizing and secreting insulin. Insulin secretion is stimulated by glucose ingestion, but nutrients like free fatty acids and amino acids can also boost glucose-induced insulin release. Moreover, several hormones such as leptin, melatonin, estrogen, growth hormone and glucagon-like peptide-1 play a role in regulating insulin secretion[4]. Insulin deficiency is known to significantly reduce leptin levels, a peptide hormone encoded by the obese gene primarily in adipocytes[5-9]. Leptin exerts its effects upon binding to receptors found in various organs, including the pancreas, brain, lung, kidney, heart, skeletal muscle, adrenal gland, spleen, intestine, bone marrow, rumen, ovary, uterus and placenta[10-13]. Despite mediating numerous physiological functions, the underlying mechanisms of leptin's actions remain incompletely explained. Nevertheless, the mechanisms and biomolecular pathways through which leptin regulates pancreatic secretions await full clarification[14,15]. Nitric oxide (NO) is a critical molecule in biological systems, serving as a fundamental signal for leptin's action in various cell types, including pancreatic β-cells, adipocytes, neurons, immune cells and muscle cells[16]. Although NO plays a role in the changes accompanying diabetes, the relationship between NO and leptin in physiological changes has not been fully elucidated in animal models of diabetes[17]. Inducible Nitric Oxide Synthase (iNOS) can be induced by cytokines in pathological conditions, resulting in inducible nitric oxide (iNO), which reinforces the body's immune response. Diabetes exhibits elevated NO production from both endothelial nitric oxide synthase (eNOS) and iNOS[18,19].

The aim of this study was to investigate the interaction between leptin and insulin with NO in the pancreatic tissue of streptozotocin (STZ)-induced diabetic rats, utilizing immunohistochemical and biochemical methods.

MATERIALS AND METHODS

Study area: The research was conducted in the Histology-Physiology Laboratory of the Biology Department, Faculty of Science, at the University of Istanbul (Türkiye) from March, 2004 to July, 2005.

Animals and experimental design: Following approval from the local Animal Experiments Ethics Committee (Decision No. 2004/34), 56 healthy Wistar albino male rats, aged 3 months and weighing between 250-300 g, were utilized in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals published by Istanbul University. The rats were housed in plastic cages under controlled room temperature conditions (22±3°C) with a 12-12 hrs light-dark cycle and they had access to commercial pellet feed and water *ad libitum* for four weeks.

The rats were randomly assigned to seven groups (n = 8) as follows: Control group: Received a single intraperitoneal (i.p.) injection of serum physiological saline only. The STZ group: The diabetic control group received a single i.p., injection of freshly prepared streptozotocin (STZ) (65 mg/kg b.wt., Calbiochem 572201) in serum physiological saline only. Leptin group: Treated daily with leptin (0.5 µg/kg, i.p., Biomol Sc-843) for three weeks, starting one week after the formation of experimental groups. Insulin group: Treated daily with insulin (20 U/kg/day; intramuscular; Humulin R, Lilly, 100 IU/mL/HI-210) for three weeks, starting one week after the formation of experimental groups. The STZ+Leptin group: Treated daily with leptin for three weeks after the induction of diabetes. The STZ+Insulin group: Treated daily with insulin for three weeks after the induction of diabetes. The STZ+Leptin+Insulin group: Treated daily with both leptin and insulin for three weeks after the induction of diabetes.

Blood glucose levels were assessed at the commencement and conclusion of the experiment using a glucometer (GlucoMen; A. Menarini Diagnostic). Rats were classified as diabetic if their random blood glucose levels equaled or exceeded 180 mg/dL.

Biochemical analysis: Blood was obtained via the heart puncture technique from each rat under Nembutal anesthesia (70 mg/kg; Ulagay, Türkiye) and centrifuged at x3000 g for 10 min to isolate serum. The serum samples were then stored at -80°C until required. Leptin levels in the serum were quantified using a radioimmunoassay (RIA) with a rat leptin kit (Linco Research, Missouri, USA). Simultaneously, insulin levels were determined utilizing an ELISA kit (Rat Insulin Kit, Mercodia, Uppsala, Sweden).

Histological analysis: The pancreas was promptly extracted for histological assessments under anesthesia. Tissue samples were fixed in a 10% neutral formalin solution for 24 hrs. A standard paraffin-embedding procedure was employed to generate 5 µm thick sections, which were subsequently

stained with aldehyde fuchsin (AF) to visualize β-cells within the islets. Morphological examinations were conducted using a Leica light microscope and images were captured utilizing Kameram 390 CU Software (Mikro Sistem Ltd., Sti, Türkiye).

Immunohistochemical analysis: The expressions of eNOS, iNOS and leptin in pancreatic tissue were identified utilizing the streptavidin-biotin complex (Strep-ABC) method. The sections were incubated overnight at 4°C with rabbit anti-mouse antibodies for eNOS (Neomarker, RB-1711-P, Fremont, California, USA), iNOS (Neomarker, RB-1605-P, Fremont, California, USA) and leptin (Ob Antibody (Y-20): sc-843, Santa Cruz Biotechnology, Texas, USA) at a 1:50 dilution. Control procedures were conducted on adjacent sections of the same tissues, replacing PBS. Both the intensity and distribution of specific eNOS, iNOS and leptin staining in Langerhans islets and the exocrine pancreas were evaluated using a semi-quantitative method, as previously described by Oztay *et al.*[19].

Statistical analysis: Statistical significance was determined through One-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test, utilizing GraphPad Prism (GraphPad Prism Version 5 Software Program, San Diego, California, USA). The results were presented as Mean±SEM (standard error of the mean). A p-value less than 0.05 was considered statistically significant.

RESULTS

Histological and immunohistochemical results: In all experimental groups, except the control group, notable histopathological alterations were observed, including increased connective tissue, enlarged blood vessels throughout the tissue, including the islets, vacuolization in the cytoplasm of both the islet cells and acini cells, invaginations of the islet periphery and disrupted acinar architecture. These changes were more prominent in the insulin group. Additionally, the islets of the STZ group did not exhibit staining with aldehyde fuchsin and the islets of the leptin group demonstrated staining similar to those of the control group, while they were weakly stained in the insulin group compared to the control group (Fig. 1).

The eNOS immunoreactivity was more pronounced in α cells at the periphery of the islets in the control group, while both β-cells in the core of each islet and acini cells displayed weaker immunoreactivity. In the leptin group, both α and β-cells exhibited stronger eNOS immunoreactivity, which was weaker in acini cells. In comparison to the control group, eNOS immunoreactivity was enhanced in α, β and acini cells of the insulin group. The STZ group displayed moderate and more diffuse eNOS immunoreactivity compared to the control group, but it was notably weak in acini cells. When compared to the STZ group, both the STZ+Leptin and STZ+Insulin groups demonstrated stronger eNOS immunoreactivity in α, β and acini cells. However, the eNOS immunoreactivity of the islets was more pronounced in the STZ+Leptin+Insulin group compared to the control group, with that of acini cells being similar to the STZ group (Table 1 and Fig. 2).

A strong iNOS immunoreactivity was observed in α cells of the control group, while it was less intense in β-cells and moderate immunoreactivity was noted in acini cells. In the leptin and insulin groups, immunoreactivity for iNOS in both α and β-cells was moderate, while acini cells exhibited weak immunoreactivity. In the STZ group, both the islets and acini cells displayed very strong iNOS immunoreactivity. The iNOS immunoreactivity was weaker in the islets and acini cells of the STZ+Leptin and STZ+Insulin groups compared to the STZ group. In the STZ+Leptin+Insulin group, weaker immunoreactivity was present in α and β-cells compared to the STZ group and iNOS immunoreactivity was nearly non-existent in acini cells (Table 1 and Fig. 2).

Table 1: Immunoreactivity scores of pancreatic tissue in the experimental groups

	Immunoreactivity scores					
	eNOS		iNOS		Leptin	
Experimental group	Langerhans islets	Acini cells	Langerhans islets	Acini cells	Langerhans islets	Acini cells
Control	++	++±	++±	++	++±	±
STZ	++±	±	++++	++++	++±	++
Leptin	++++	++	++±	++	+±	-
Insulin	+++	+++	++	+	++±	+
STZ+Leptin	+++	+++	+++	++±	+	±
STZ+Insulin	+++±	++++	++±	+++	++	+
STZ+Leptin+Insulin	+++	+	++	±	++±	±

Intensity of eNOS, iNOS and leptin immunoreactivity for each group is represented by the number of plus signs. Immunoreactivity intensities linearly increase from weak (+) to strong (++++)

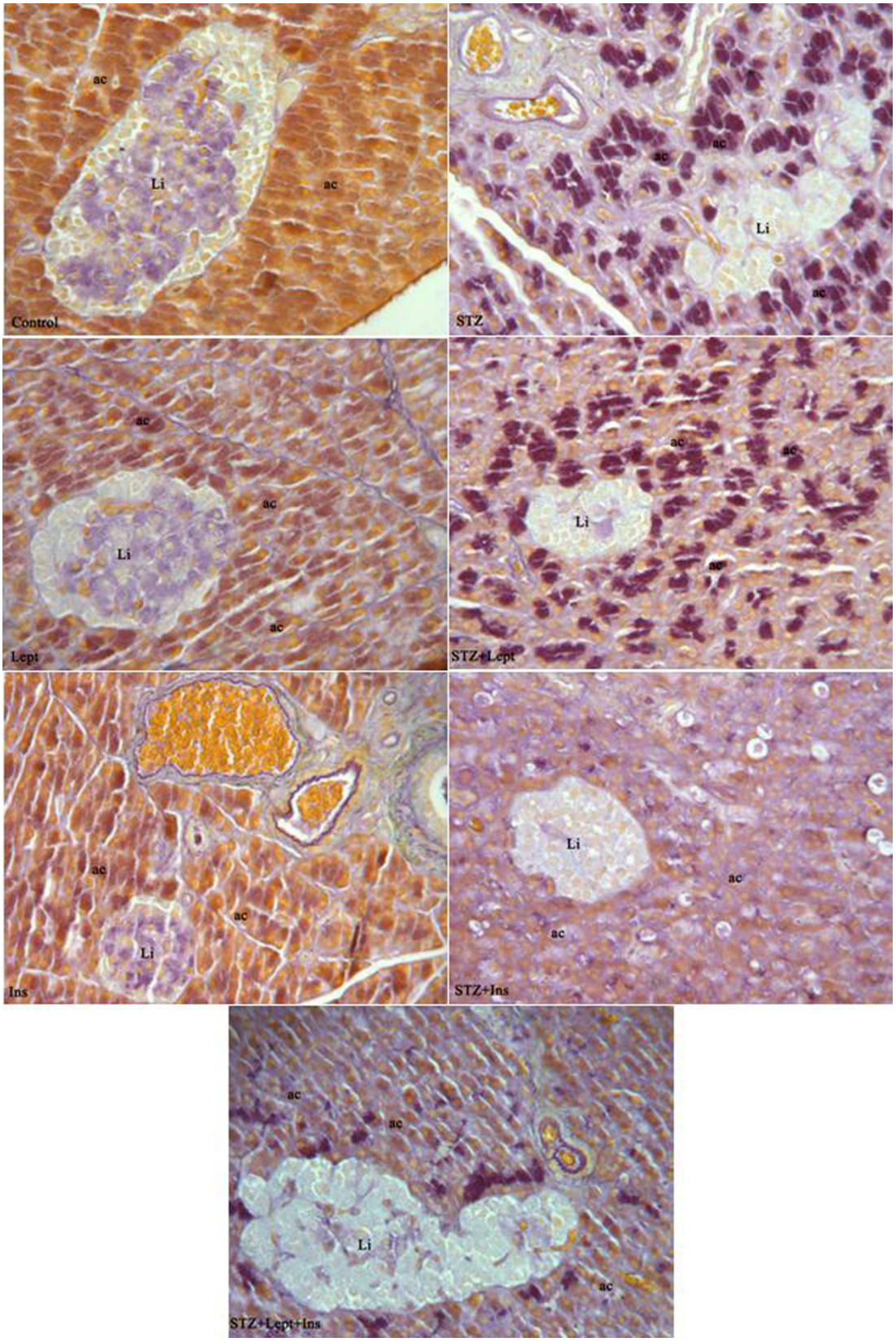

Fig. 1: Representative images of histological changes in all groups revealed by aldehyde fuchsin staining
Li: Langerhans islet, ac: Acini cells and x200 magnification

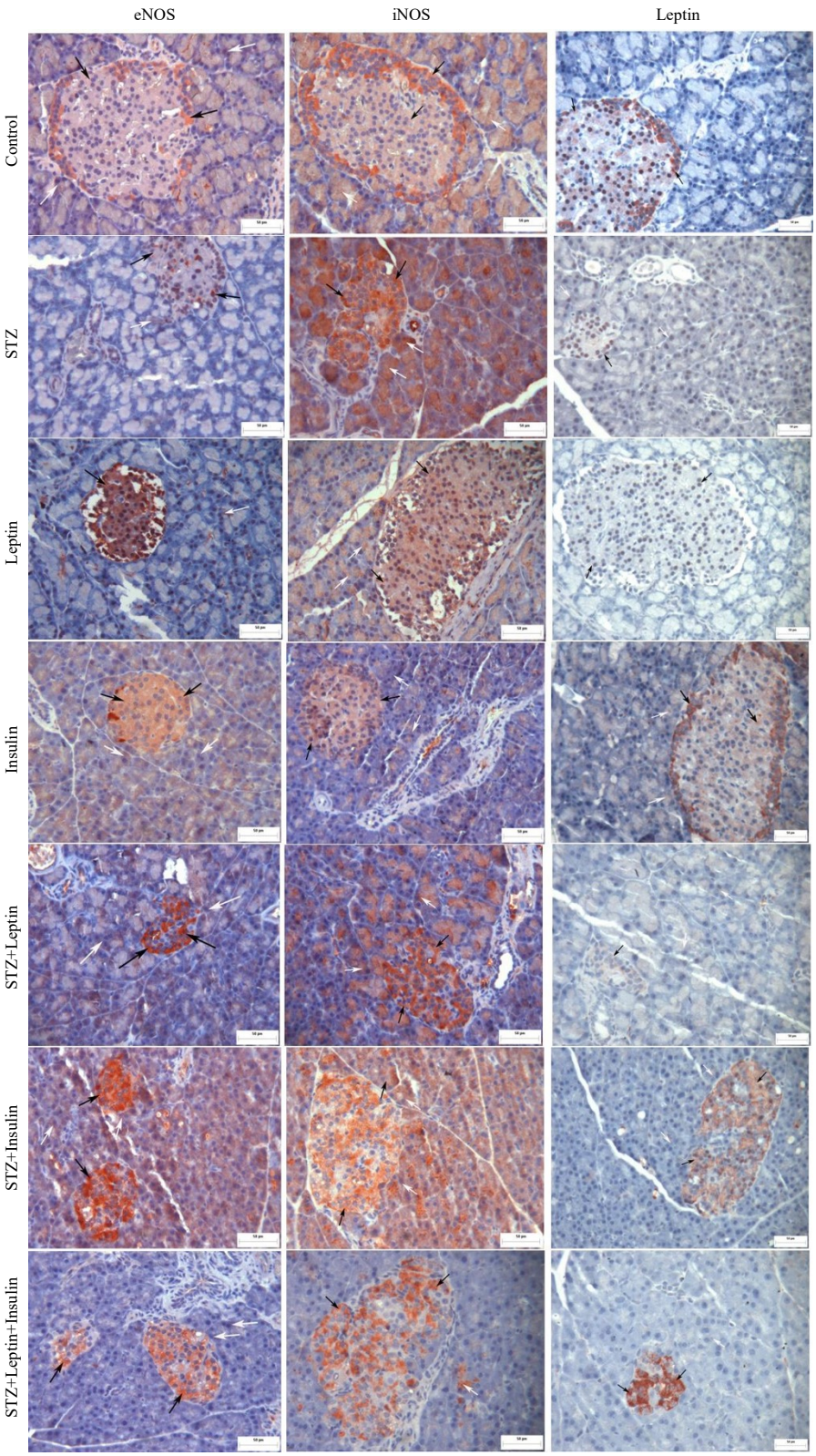

Fig. 2: Representative images of immunohistochemical distribution and expression of eNOS, iNOS and leptin in all groups (indicated with ↑)
Bar: 50 µm

Table 2: Levels of blood glucose (mg/dl), serum leptin and insulin in the experimental groups

Experimental group	Initial glucose levels (mg/dL)	Final glucose levels (mg/dL)	Serum leptin levels (ng/mL)	Serum insulin levels (µg/L)
Control	102.7±1.5	93.7±1.7	2.9830±0.2574	2.517±0.282
STZ	98.7±4.0	313.5±40.2***	0.5864±0.0907***	0.633±0.067***
Leptin	104.7±3.7	81.7±1.4	2.0440±0.1392***	1.895±0.135*
Insulin	101.3±3.6	40.7±6.3	0.0240±0.0013***	1.300±0.026***
STZ+Leptin	100.7±2.3	304.8±46.0***	0.0102±0.0003***,+	0.624±0.048***
STZ+Insulin	102.0±3.1	114.0±10.4+++	0.0012±0.0001***,+	1.267±0.054***,+
STZ+Leptin+Insulin	97.5±1.2	118.3±10.9+++	0.1150±0.0022***	1.900±0.073*,+++

*p<0.05, ***p<0.001 compared to the control group and +p<0.05, +++p<0.001 compared to the STZ group

Leptin immunoreactivity was evident in both α and β-cells, albeit very weak in acini cells in the control group. In the STZ group, the intensity of immunoreactivity was moderate in both the islets and acini cells. The leptin group exhibited positive immunoreactivity in the islets, albeit weaker compared to the control group, with no immunoreactivity observed in acini cells. In the insulin group, immunoreactivity was stronger at the periphery of the islets and weaker in acini cells compared to the control group. The STZ+Leptin group, where the islets were disrupted, displayed pale immunostaining in both islet cells and acini cells compared to the control and STZ groups. The STZ+Insulin group presented weaker leptin immunoreactivity in the islets, acini cells and ducts compared to the STZ group. A notable decrease in leptin immunostaining was observed in the STZ+Leptin group compared to the STZ+Insulin group. In contrast, the STZ+Leptin+Insulin group showed intense positive immunoreactivity for leptin in the islets, with a rather weak immunoreactivity in acini cells (Table 1 and Fig. 2).

Biochemical results: Initially, the blood glucose levels were similar across all groups. However, at the end of the experiment, the STZ and STZ+Leptin groups exhibited a significant increase in blood glucose levels ($p<0.001$) compared to the control group. Conversely, the blood glucose levels of the STZ+Insulin and STZ+Leptin+Insulin groups closely resembled those of the control group. The insulin group showed a decrease in blood glucose levels, although it was not statistically significant (Table 2). The serum leptin and insulin levels in the STZ group were significantly lower than those in the control group ($p<0.001$ for both). Leptin levels were markedly reduced in the leptin and insulin groups compared to the control group ($p<0.001$), with the insulin group exhibiting lower levels than the leptin group. Despite the considerably lower leptin levels in the STZ+Leptin and STZ+Insulin groups, there was a partial increase in these levels in the STZ+Leptin+Insulin group. Serum insulin levels decreased in all groups compared to the control group ($p<0.001$ for the STZ, Insulin, STZ+Leptin and STZ+Insulin groups; $p<0.05$ for the Leptin and STZ+Leptin+Insulin groups). The insulin levels in the leptin group were closer to those in the control group, while the STZ+Leptin group exhibited lower levels. However, the insulin and STZ+Insulin groups showed quite similar insulin levels. Notably, insulin levels increased in the STZ+Leptin+Insulin group, approaching those of the control group (Table 2).

DISCUSSION

The STZ, an antibiotic produced by *Streptomyces achromogenes*, is widely employed to induce experimental models of DM in various animal species. Its selective damage to pancreatic β-cells results in subsequent diabetes development. Notably, the damage inflicted in male rats is markedly higher than in females, prompting the selection of male rats as model animals in this study, consistent with the approach adopted by Rifaai *et al.*[1] and Wu *et al.*[20]. The STZ-induced rat model of DM closely mirrors human diabetes, exhibiting analogous pathological and physiological changes. Consequently, findings from studies utilizing this experimental model offer valuable insights for designing new adjunctive therapies targeting pancreatic disorders, including DM.

Both Neuronal Nitric Oxide Synthase (nNOS) and eNOS isoforms play pivotal roles in regulating both endocrine and exocrine secretion in the pancreas under normal physiological conditions. Notably, eNOS stands out as the primary isoform in pancreatic regulation. However, in pathologies like DM and pancreatitis, inflammatory processes are predominantly triggered by iNOS rather than eNOS[1,21-23]. This study specifically focused on investigating these two isoforms, recognizing the distinctive contributions of nNOS and eNOS in normal pancreatic function and the crucial role of iNOS in inflammatory pathways associated with DM and pancreatitis.

The presence and localization of nitric oxide synthases in the pancreas have been a subject of contention. Some studies exclusively detect all three NOS isoforms in pancreatic islets, with no presence in the exocrine part, while others report detectable levels of NOS in pancreatic acini[23,24]. In the current study, varying degrees of endothelial NOS (eNOS) and Inducible NOS (iNOS) immunoreactivity, ranging from weak to very strong, in both pancreatic islets and acini of both normal and diabetic rats were observed. Consistent with the findings of the current study, Buchwalow *et al.*[23] detected all three NOS isoforms in both exocrine and endocrine parts of the normal and inflamed pancreas. The controversy surrounding the presence and localization of NOS in the pancreas may, as suggested by Buchwalow *et al.*[23], reflect the diversity of experimental approaches and potential limitations in the sensitivity of the methods employed.

The DM is linked to oxidative stress induced by free radicals, a consequence of pancreatic cell damage and tissue degeneration. The NO radical, generated by NOS isoforms, is considered a potential mediator of pancreatic β-cell damage in streptozotocin (STZ)-induced DM[1,23,25,26]. However, it is noteworthy that NO might not be the sole molecule responsible for the damage induced by STZ in diabetic pancreas, as indicated by several studies[27]. Although the precise mechanism remains unclear, studies suggest that eNOS overexpression exerts a protective effect against chemically induced diabetes[28,29]. Rifaai *et al.*[1] observed strong immunostaining for iNOS in inflammatory cells infiltrating islets, the endothelium of blood vessels and some acinar cells in STZ-induced diabetic rats compared to control rats. In alignment with these observations, the current study revealed strong to very strong immunostaining for both eNOS and iNOS in Langerhans islets and acinar cells of the diabetic experimental groups.

Leptin, a peptidic hormone comprising 167 amino acids, plays a multifaceted role in regulating gastric and pancreatic secretions, insulin release and mitigating inflammatory processes in the pancreas[30]. Notably, pancreatic β-cells emerge as a crucial target for this hormone, as it directly inhibits insulin synthesis and release from these cells[31,32]. The study by Dall'Aglio *et al.*[33] demonstrated the immunolocalization of leptin throughout the pancreatic islets, leading to the conclusion that all endocrine cells may have the capacity to secrete this hormone. Observation of stronger leptin immunoreactivity in Langerhans islets compared to acinar cells in the present study aligns with their findings, providing further support to the notion that leptin may have a pronounced presence and impact within pancreatic islets.

Leptin therapy has been demonstrated to diminish insulin secretion in ob/ob mice, rats and humans by suppressing insulin production and release from β-cells. Conversely, insulin plays a role in stimulating the adipose tissue to produce and secrete leptin[15]. The delicate balance between insulin from β-cells and leptin from adipose tissue is crucial for maintaining[34].

Exogenous leptin is known to significantly reduce blood glucose levels in both control and diabetic rats[35]. However, in our study, while leptin treatment effectively lowered blood glucose levels in control animals, it had no significant impact on diabetic rats, maintaining their blood glucose levels close to those of the diabetic control group. These disparities are likely attributed to variations in experimental conditions. In insulin-deficient rodent diabetic models, leptin therapy may lower blood glucose by increasing insulin levels or promoting β-cell regeneration. Additionally, it could enhance insulin sensitivity in type 1 diabetes models[36,37]. Denroche *et al.*[38] reported a dose-dependent glucose-lowering effect of leptin in streptozotocin (STZ)-diabetic mice. Oztay *et al.*[19] suggested that the duration of the experiment might account for a slight decrease in blood glucose levels observed in their study with leptin treatment.

In our experiments, the co-administration of leptin and insulin to diabetic rats significantly influenced blood glucose levels, as well as serum leptin and insulin concentrations, effectively restoring blood glucose levels closer to those of the control group. The requirement of insulin for the glucose-lowering effect of leptin has been supported by several studies, indicating that leptin can potentially enhance insulin sensitivity[15,35]. The heightened leptin expression observed in our findings in the combination group may be linked to improved insulin sensitivity. Moreover, in this study, iNOS immunoreactivity was notably weakest in the diabetic group that received the co-administration of leptin and insulin, suggesting that leptin may exert its effects through the suppression of iNOS.

Konturek *et al.*[39] observed that exogenous leptin confers protection to the pancreas against the development of acute pancreatitis induced by caerulein, a ten-amino acid oligopeptide, through the activation of the NO pathway.

CONCLUSION

The present study demonstrated that leptin treatment, when administered in conjunction with insulin, normalizes hyperglycemia in diabetic rats. This normalization is achieved by suppressing iNOS availability and enhancing insulin sensitivity in the pancreas. However, to gain a more comprehensive understanding of the relationship among NO, leptin and insulin in the diabetic pancreas, further studies should be conducted.

SIGNIFICANCE STATEMENT

In addition to various hormones, leptin also regulates insulin secretion. Leptin levels are significantly reduced in insulin deficiency, leading to diabetes. The relationship between nitric oxide (NO) and leptin has not been fully elucidated in animal models of diabetes. The NO generation by both Endothelial Nitric Oxide Synthase (eNOS) and Inducible Nitric Oxide Synthase (iNOS) increases in diabetes. This study investigated the interaction of leptin and insulin with NO in the pancreatic tissue of streptozotocin (STZ)-induced diabetic rats. To our knowledge, this is the first report demonstrating that combined therapy with leptin and insulin normalizes hyperglycemia in diabetic rats by suppressing iNOS availability and increasing insulin sensitivity in the pancreas.

ACKNOWLEDGMENT

This work was supported by Scientific Research Projects Coordination Unit of Istanbul University [grant number: 281/30092004].

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