HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

# The effect of Hydroxytyrosol on Spexin immunoreactivity in pancreatic islets and serum insulin levels in a Streptozotocin-induced experimental diabetes model in rats

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**Abstract:** Diabetes (DM), a major health problem worldwide, is associated with the loss of β cells in the pancreatic islets and decreased insulin secretion. Hydroxytyrosol (HxT) is a phenol found in high concentrations in olive oil. Spexin (SPX) plays a role in regulating many metabolisms such as glucose and energy. This study aimed to determine the effects of HxT on circulating insulin levels, histopathological changes in pancreatic islets, and SPX immunoreactivity in a Streptozotocin-induced experimental diabetes model (eDM) in rats.

The 32 male rats used in the study were randomly divided into 4 groups (n: 8): Control, eDM, eDM+HxT, and HxT. After completing all applications in the experiment, the blood and pancreas tissues of the sacrificed rats were taken. Insulin levels were determined from the serum samples obtained. Histopathological changes and SPX immunoreactivities were evaluated in pancreatic tissues.

While serum insulin levels decreased in eDM, histopathological changes in pancreatic islets increased. Additionally, SPX immunoreactivity in pancreatic islets was significantly reduced in eDM. On the other hand, HxT supplementation (eDM+HxT group) regulated eDM-related adverse effects. While HxT supplementation may have a curative and therapeutic effect in DM, it was concluded that SPX may be effective in regulating the endocrine functions of the pancreas.

***Keywords:*** *Diabetes, Hydroxytyrosol, Pancreas, Spexin, Streptozotocin.*

## Sıçanlarda Streptozotosin ile İndüklenen Deneysel Diyabet Modelinde Hidroksitirosol'ün Pankreas Adacıklarında Speksin İmmünoreaktivitesi ve Serum İnsülin Düzeyleri Üzerine Etkisi

**Özet**:Dünya çapında önemli bir sağlık sorunu olan diyabet (DM), pankreas adacıklarındaki β hücrelerinin kaybı ve insülin sekresyonunun azalmasıyla ilişkilidir. Hidroksitirosol (HxT), zeytinyağında yüksek konsantrasyonda bulunan bir fenoldür. Spexin (SPX), glikoz ve enerji gibi birçok metabolizmanın düzenlenmesinde rol oynar. Bu çalışma, sıçanlarda Streptozotosin ile indüklenen deneysel diyabet modelinde (eDM) HxT'nin dolaşımdaki insülin düzeyleri, pankreas adacıklarındaki histopatolojik değişiklikler ve SPX immünoreaktivitesi üzerindeki etkilerini belirlemeyi amaçladı.

Çalışmada kullanılan 32 adet erkek sıçan, Kontrol, eDM, eDM+HxT ve HxT olmak üzere rastgele 4 gruba (n:8) ayrıldı. Deneyde tüm uygulamaların tamamlanmasıyla sakrifiye edilen sıçanların, kan ve pankreas dokuları alındı. Elde edilen serum örneklerinden insülin düzeyleri tespit edildi. Pankreas dokularında ise histopatolojik değişiklikler ve SPX immünreaktiviteleri değerlendirildi.

Serum insülin düzeyleri eDM'de azalırken pankreas adacıklarında histopatolojik değişikliklerin arttığı görüldü. Ayrıca eDM'de pankreas adacıklarında SPX immünreaktivitesi önemli ölçüde azaldığı tespit edildi. Buna karşın HxT takviyesinin (eDM+HxT grup), eDM'ye bağlı olumsuzlukları ortadan kaldırdığı görüldü.

DM'de HxT takviyesi iyileştirici ve tedavi edici etki gösterebilirken, SPX'in pankreasın endokrin fonksiyonlarını düzenlemede etkili olabileceği kanaatine varıldı.

***Anahtar Kelimeler:*** *Diyabet, Hidroksitirozol, Pankreas, Spexin, Streptozotosin.*

## Introduction

Over half a billion people globally have been diagnosed with diabetes (DM), a severe health issue whose prevalence is alarmingly high. Type 2 DM (T2DM), the most prevalent kind, is distinguished by persistent hyperglycemia. This is the primary pathological mechanism causing tissue damage and long-term complications (Binou et al., 2023). At the beginning of T1DM, there is a noticeable lack of β-cells. Similar circumstances are seen in the latter stages of T2DM (Lytrivi et al., 2020).

Among various nutritional models, the Mediterranean diet has been reported to positively affect DM and its complications (Estruch et al., 2018). Bioactive substances found in abundance in the Mediterranean diet include polyphenols, monounsaturated fatty acids, and olive oil (Vlavcheski et al., 2019). As a byproduct of the body's metabolism of oleuropein, hydroxytyrosol (HxT) is the phenol present in olive oil at the highest concentration (Vlavcheski et al., 2019). Research indicates that HxT influences lipid and glucose homeostasis, has antioxidant qualities, and may be protective against DM (Alkhatib et al., 2018; Wani et al., 2018).

SPX is a recently identified peptide hormone (Yalcin et al., 2024). Along with promoting glucose and lipid metabolism, SPX has various pleiotropic metabolic effects, such as modulating insulin secretion and energy homeostasis (Kaya et al., 2023). It has been proposed that only mammalian metabolically active organs and tissues , such as the pancreas, liver, stomach, and adipose tissue, secrete SPX (Lv et al., 2019). Furthermore, it is known that hormones like insulin, glucagon, estradiol, and adrenocorticotropic can alter the secretion of SPX in rodents (Wang et al., 2020). Notably, prior research has shown that SPX and insulin co-localize in the secretory vesicles of pancreatic β cells (Gallagher et al., 2024; Gowdu and Dayanand, 2021). This suggests that there is a connection between SPX and insulin and that they may interact (Kaya, 2023a).

This study aimed to examine the effects of HxT on histopathological and SPX immunoreactivity in pancreatic islets and to determine circulating insulin levels in a streptozotocin (STZ)-induced experimental diabetes model (eDM) in rats.

## Material and Method

**Design of Experiments:** This study was carried out with the approval of the Fırat University Experimental Animals Local Ethics Committee, with its decision dated 13.11.2023 and numbered 19739. The experimental design and applications of the study were carried out within the scope of ARRIVE guidelines. The 32 Sprague-Dawley rats (male, 200-230 g, 8-10 weeks old) used in the study were kept under optimal conditions (22-25 °C temperature, 12-hour light cycle, ad-libitum water, and pellet feed). With eight rats per group, the rats were split into four groups at random. No app was made to the control group (n:8) during the experiment. To the eDM group (n: 8), a single dose of 50 mg/kg STZ (dissolved in phosphate-citrate buffer (pH4.5), Sigma Chemical) was administered intraperitoneally to create experimental DM. 72 hours after STZ administration, blood samples (tail vein) were taken from fasted rats for 12 hours, and blood glucose levels were measured. eDM was diagnosed in rats with fasting blood glucose levels over 250 mg/dL. To the eDM+HxT group (n:8), 5 mg/kg/day HxT (Sigma Chemical) was administered via an orogastric tube for six weeks after experimental DM was created with STZ. To the HxT group (n: 8), 5 mg/kg/day HxT was administered via an orogastric tube during the 6-week experiment. The STZ and HxT doses and applications used in the experimental design were determined, taking into account previous studies (Rodriguez-Pérez et al., 2022; Rodriguez-Pérez et al., 2023). After all applications were completed in the trial, the rats were sacrificed by decapitation method under anesthesia (xylazine 10 mg/kg + ketamine 75 mg/kg intraperitoneally), and blood and pancreatic tissues were received.

**Histopathological and Immunohistochemical evaluations:** Pancreatic tissues taken at the end of the experiment were fixed in 10% formalin solution and subjected to a histological follow-up series. Pancreatic tissue sections were stained using Hematoxylin Eosin (HE) for histopathological analyses. The degeneration of pancreatic islets was taken into account during the histopathological evaluation of pancreatic tissues. Histopathological histoscorography was created by evaluating 10 separate, non-overlapping areas at X20 magnification from 2 HE-stained pancreatic tissue sections prepared for each rat. In the histopathological evaluation of pancreatic tissue, a 3-grade scoring system was used according to the presence of degeneration of pancreatic islets (none; 0, mild; 1, moderate; 2 and severe; 3) (Lee et al., 2016; Uyar and Abdulrahman, 2020). The avidin-biotin-peroxidase complex method (Kaya, 2023b) was used to determine SPX (1:200, Boster Bio-Tech, A04088-1, USA) immunoreactivity in pancreatic tissues. Counterstaining was performed with Mayer Hematoxylin. SPX immunoreactivities in pancreatic tissue were calculated with 3-grade scoring (none; 0, mild; 1, moderate; 2 and severe; 3) (Lee et al., 2016; Uyar and Abdulrahman, 2020). Histopathological and immunoreactivity evaluations of all prepared preparations were made under a light microscope and microphotographs were taken (Leica, DM 2500, Germany).

**Biochemical analysis:** Blood samples taken after the experiment were centrifuged, and serum was taken. The enzyme-linked immunosorbent assay (ELISA) was used to measure Insulin levels using blood serum samples. Insulin (ER1113, FineTest, Wuhan, China) ELISA kit purchased from the commercial company was studied considering the manufacturer's instructions. The sensitivity of the insulin ELISA kit was 46.875 pg/ml and the test range was 78.125-5000 pg/ml. Additionally, an automatic biochemical analyzer (ADVIA-2400, Siemens) was used to measure the amount of glucose in blood serum.

**Statistical analysis:** The study data was analyzed using the SPSS 22.0 package program. Data showing homogeneous

distribution according to the Shapiro-Wilk test were statistically analyzed using Oneway ANOVA posthoc TUKEY tests. For a p-value <0.05, statistical significance was considered. The statistical analysis results of the study data were presented in graphs created using Graph Pad Prism 9.3 software.

## Results

**Effect of eDM and/or HxT on histopathological and SPX immunoreactivity in pancreatic tissue:** Pancreatic tissue and islets were observed to have typical histological structures in the control and HxT groups. Atrophic degenerate islets were found to be more common in the pancreatic islets in the eDM group compared to the control group (P<0.05). In the eDM+HxT group, HxT administration significantly reduced the degenerated pancreatic islets observed in the eDM model compared to the eDM group (P<0.05) (Figure 1).

SPX immunoreactivities in pancreatic islets were at similar levels in the control and HxT groups (P>0.05). SPX immunoreactivity in pancreatic islets was decreased in the eDM group compared to the control group (P<0.05). On the other hand, SPX immunoreactivity in pancreatic islets augmented in the eDM+HxT group compared to the eDM group (P<0.05) (Figure 2).

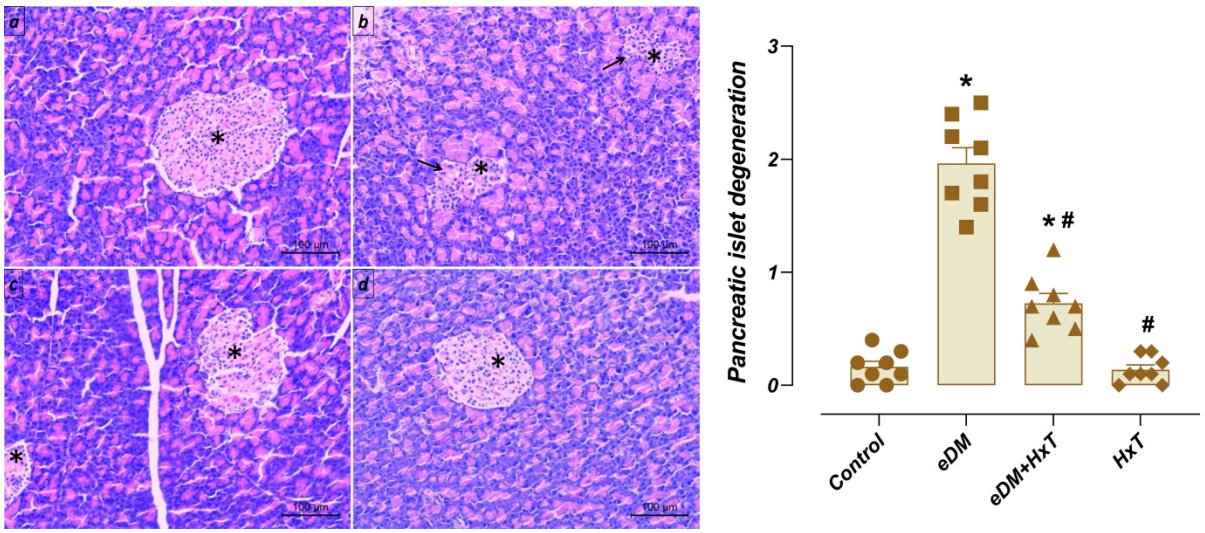


**Figure 1. Microphotographs and histopathological histoscore graph of the histopathological effects of eDM and/or HxT on pancreatic islets.** Control (a) and HxT (d) groups had normal histological pancreatic tissue. Widespread degeneration was observed in the pancreatic islets of the eDM (b) group compared to the control group (P<0.05). In the eDM+HxT (c) group, it was determined that the degeneration observed in the pancreatic islets was significantly reduced compared to the eDM group (P<0.05). Hematoxylin Eosin staining, scale bar; 100µm. Star; pancreatic islets, arrow; pancreatic islet degeneration. Histopathological histoscore graph: *; compared to control (P<0.05), #; Compared to eDM (P<0.05). eDM; experimental diabetes model, HxT; Hydroxytyrosol.

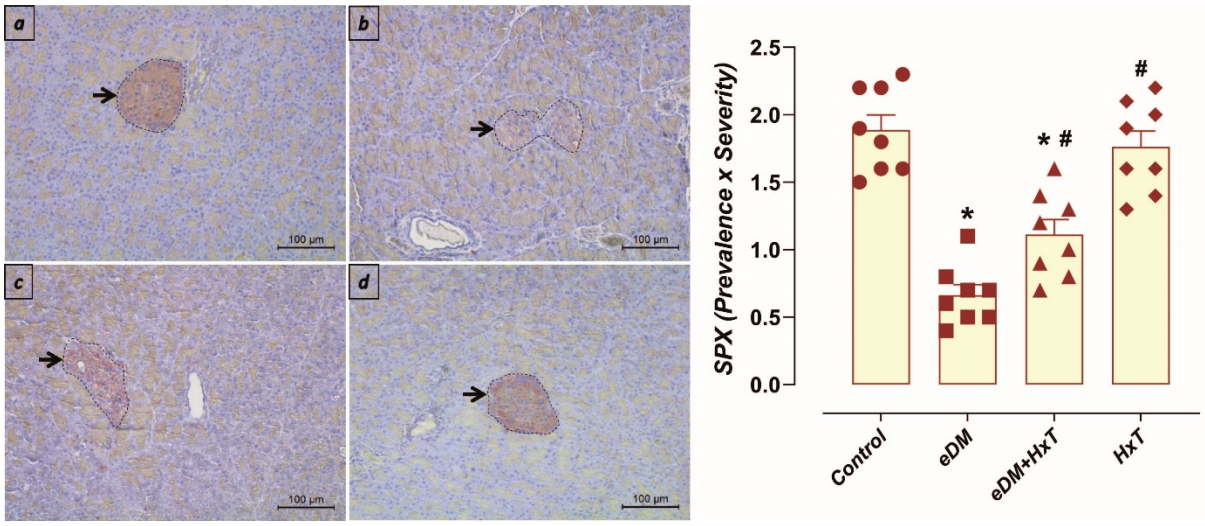


**Figure 2. Microphotographs and immunoreactivity graph of the effects of eDM and/or HxT on pancreatic islets SPX immunoreactivity.** SPX immunoreactivity was similar in pancreatic islets in the control (a) and HxT (d) groups. It was observed that SPX immunoreactivity decreased in the pancreatic islets of the eDM (b) group compared to the control group (p<0.05). In the eDM+HxT (c) group, it was determined that SPX immunoreactivity increased in the pancreatic islets compared to the eDM group (P<0.05). SPX immunohistochemical staining, scale bar; 100µm. Arrow; SPX immunoreactivity in pancreatic islets. Immunoreactivity graph: *; compared to control (P<0.05), #; compared to eDM (P<0.05). eDM; experimental diabetes model, HxT; Hydroxytyrosol, SPX; Spexin.

**Effect of eDM and/or HxT on serum insulin and glucose levels:** There was no difference between the insulin levels of the control and HxT groups in blood serum samples (P>0.05). It was detected that insulin levels in the eDM group were reduced compared to the control group (P<0.05). Insulin levels were found to be increased in the eDM+HxT group compared to the eDM group (P<0.05) (Figure 3).

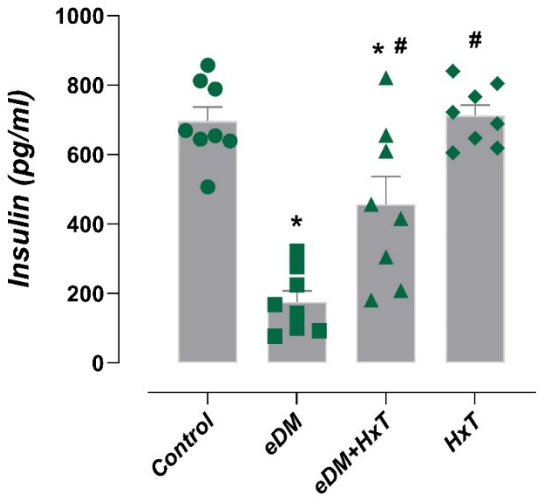


**Figure 3. Effects of eDM and/or HxT on serum insulin levels.** Serum insulin levels were similar in the control and HxT groups. It was determined that serum insulin levels decreased in the eDM group compared to the control group (P<0.05). Serum insulin levels increased in the eDM+HxT group compared to the eDM group (P<0.05). *; compared to control (P<0.05), #; compared to eDM (P<0.05). eDM; experimental diabetes model, HxT; Hydroxytyrosol.

Blood glucose levels were similar between the control and HT groups (P>0.05). When the eDM group was compared with the eDM+HxT group, glucose levels were observed to increase (P<0.05). It was determined that glucose levels decreased in the eDM+HxT group compared to the DM group (P<0.05) (Figure 4).

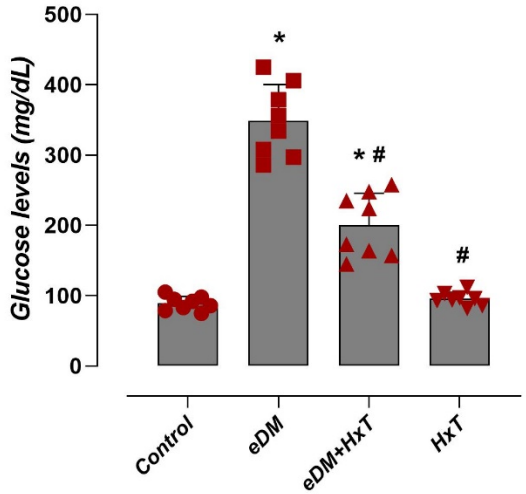


**Figure 4. Effects of eDM and/or HxT on serum glucose levels.** Serum glucose levels were similar in the control and HxT groups. It was determined that serum glucose levels increased in the eDM group compared to the control group (P<0.05). Serum glucose levels were found to decrease in the eDM+HxT group compared to the eDM group (P<0.05). *; compared to control (P<0.05), #; compared to eDM (P<0.05). eDM; experimental diabetes model, HxT; Hydroxytyrosol.

## Discussion and Conclusion

This study investigated the effects of HxT in eDM on circulating insulin levels, SPX immunoreactivity, and histopathological changes in pancreatic islets. HxT administration to eDM rats increased SPX immunoreactivity while reducing histopathological changes compared to the eDM group. Additionally, HxT administration to rats with eDM increased circulating insulin levels in parallel with this increase in SPX immunoreactivity in pancreatic islets compared to the eDM group.

One study found that oral administration of 5, 50, and 500 mg/kg/day HxT to rats for 13 weeks had no negative effects on micro/macro-organ changes, morbidity, or mortality (Auñon-Calles et al., 2013). HxT is crucial in preventing DM (Bertelli et al., 2020). This study determined that pancreatic islets underwent STZ-induced atrophic degeneration and largely lost their cellular structure. However, HxT supplementation in eDM significantly reduced atrophy/degeneration in pancreatic islets. Consistent with these results, it has been reported that HxT supplementation has a protective effect on pancreatic tissue and prevents the damage of β cells (Xie et al., 2018). It has also been reported that HxT stimulates the proliferation of pancreatic β cells (Marrano et al., 2021).

In the development of DM, controlling blood glucose levels not only regulates disease-altered metabolic profiles but also reduces the incidence of complications (Rodríguez-Pérez et al., 2022). The high blood glucose levels observed in eDM in rats may results from insulin deficiency due to STZ-induced depletion of pancreatic β cells or an increased rate of gluconeogenesis and glycogenolysis (Sukanya et al., 2020). Similarly, current study observed that insulin levels decreased and glucose levels increased in the eDM group. These results were consistent with studies reporting that blood glucose levels increased in an eDM model induced by STZ injection in rats (Soylu and Karacor, 2023; Jafari-Rastegar et al., 2023). However, it has been reported that tyrosol, which is also found in olive oil, increases insulin secretion from remaining pancreatic β cells in rats with STZ-induced eDM, which may increase glucose utilization in peripheral tissues (Chandramohan et al., 2017; Jafari-Rastegar et al., 2023). Similarly, this study determined that insulin levels increased and glucose levels decreased in the eDM+HxT group compared to the eDM group.

Insulin biosynthesis and secretion are inhibited by STZ-induced NAD+ depletion in β cells (Lenzen, 2008). Additionally, STZ causes cytotoxicity and death in β cells (Soylu and Karacor, 2023). Similarly, this study detected that circulating insulin levels were reduced in the STZ-induced eDM and eDM+HxT groups compared to the control group. However, it has been reported that HxT plays an important role in insulin signaling (Wang et al., 2018). When HxT was applied to rat pancreatic tissue, the decrease in insulin secretion caused by hyperglycemia was suppressed. This suggests that HxT can increase pancreatic insulin secretion in hyperglycemic states (Hamden et al., 2009). It has been demonstrated that in β cells (rat INS-1E), HxT promotes proliferation and raises the amount of insulin protein

(Marrano et al., 2021). Consistent with these results, it was determined in this study that HxT significantly increased insulin levels, which decreased eDM. Similarly, a recent study showed that HxT increased STZ-induced decreased insulin expression (Soylu and Karacor, 2023). An another study has shown that insulin and SPX immunopositive cell numbers are significantly reduced after the onset of DM (Adeghate et al., 2022). Similarly, in this study, SPX immunoreactivity was reduced in eDM pancreatic islets. Another immunohistochemistry analysis reported that SPX has the same localization as insulin in the pancreas and that islet β cells can produce SPX (Sassek et al., 2019). Therefore, it is conceivable that circulating SPX levels are closely related to SPX expression in islet β cells. In this context, studies conducted on patients with DM have reported that there is a decrease in serum SPX levels (Dai et al., 2023; Gu et al., 2022). A study reported that the decrease in serum SPX levels due to DM may be due to the decrease in β cell mass (Dai et al., 2023). In this study, histopathological evaluation results revealed that pancreatic islets degenerate with DM, and therefore SPX immunoreactivity decreases. However, HxT treatment not only increased serum insulin levels, which decreased due to DM, but also had a regulatory effect on histopathological changes in pancreatic islets and SPX immunoreactivity.

In conclusion, STZ-induced eDM in rats increased histopathological changes in pancreatic islets and also reduced SPX immunoreactivity. Additionally, serum insulin levels were observed to decrease and glucose levels to increase in eDM. On the other hand, it has been determined that HxT supplementation regulates eDM-induced changes. These results are essential for the development of new strategies to prevent and/or treat DM, which continues to be a global health problem. Additionally, it has been suggested that SPX plays a role in the regulation of endocrine pancreatic functions.

## Similarity ratio

We declare that the similarity rate of the article is 14% as stated in the report uploaded to the system.

## Conflict of Interest

The authors did not report any conflict of interest or financial support.

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## Ethical Approval

This study was conducted with the approval of the Fırat University Animal Experiments Ethics Committee dated 13/11/2023-19739.

## Author Contributions

Motivation / Concept: TY, SK
Design: TY
Control/Supervision: SK
Data Collection and/or Processing: TY, SK
Analysis and/or Interpretation: SK, TY
Literature Review: TY
Writing the Article: TY, SK
Critical Review: SK, TY

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