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NEÜ YAYINEVİ

The Effects of Topiramate on Methotrexate-Induced Pancreatic Injury

Topiramatın Metotreksat İlişkili Pankreas Hasarı Üzerine Etkisi

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Öz

Amaç: Bu çalışmada metotreksat ilişkili pankreas hasarında topiramatın pankreasın Langerhans adacık hücreleri üzerine olan etkisinin araştırılması amaçlandı.
Gereçler ve Yöntem: Çalışma 04 Temmuz- 24 Temmuz 2022 tarihinde Recep Tayyip Erdoğan Üniversitesi Hayvan Deneyleri ünitesinde gerçekleştirilmiştir. Çalışmada Spraque-Dawley erkek sıçanlar her grupta 8 hayvan olacak şekilde 3 gruba ayrıldı. Kontrol grubuna ait sıçanlara sadece %0.9'luk serum fizyolojik intraperiteonal (i.p.) olarak uygulandı. Metotreksat (MTX) grubuna tek doz 20mg/kg MTX uygulandı. Topiramat (TPM) tedavi grubuna MTX uygulamasından 7 gün önce ve 7 gün sonra olmak üzere toplam 14 gün süreyle günde tek doz TPM 100 mg/kg/gün oral gavaj yoluyla uygulandı. TPM uygulamasından 7 gün, son dozdan 16 saat sonra tüm denekler 50 mg/kg ketamin HCL ve 20 mg/kg ksilazin i.p. uygulanarak uyutuldu.
Bulgular: MTX grubunda Langerhans adacıklarında yaygın ödematöz alanlar ve nekrotik hücreler gözlemledik. TPM grubunda MTX grubuna kıyasla nekrotik hücrelerin ve ödematöz alanların azaldığını saptadık. MTX grubunda insulin pozitivitesi gösteren β hücrelerinin kontrol grubuna kıyasla azalmış olduğunu saptadık ($p<0.05$). TPM grubundaki pankreas dokusuna ait kesitlerde MTX grubuna kıyasla insülin pozitivitesi gösteren β hücrelerinde anlamlı düzeyde artış olduğunu gözlemledik ($p<0.05$). MTX grubunda kontrol grubuna kıyasla glukagon pozitivitesi gösteren hücrelerin sayısında anlamlı olarak azalma olduğunu izledik ($p<0.05$). TPM grubunda MTX grubuna kıyasla glukagon pozitivitesini gösteren α hücrelerinin sayısının artmış olduğunu gözlemledik ($p<0.05$). Histopatolojik Hasar Skoru kontrol grubunda 0(0-1), MTX grubunda 6(6-8)'ya yükseldiğini saptadık ($p<0.05$). TPM grubunda HHS 2(2-3) olarak gözlemlendi ($p<0.05$).
Sonuç: Çalışmamız topiramatın pankreas adacık hücreleri üzerinde koruyucu bir etkiye sahip olduğunu göstermektedir.

Anahtar Kelimeler: Metotreksat, pankreas hasarı, sıçan, topiramat

Abstract

Aim: The present study aimed to investigate the effects of topiramate on pancreatic islets of Langerhans cells in Methotrexate-related pancreatic injury.
Materials and Methods: The study was conducted between 04 July - 24 July 2022 in the Animal Research Unit of Recep Tayyip Erdogan University. Male Spraque-Dawley rats were divided into 3 groups with 8 animals in each group. Control group (C) (Only 0.9% saline was administered). A single dose of 20mg/kg Methotrexate was administered to the MTX group (MTX). The TPM group (TPM) was administered 100 mg/kg/day Topiramate by oral gavage for a total of 14 days, 7 days before and after Methotrexate administration. All subjects were euthanized by anesthesia 16 hours after the last Topiramate dose.
Results: We observed widespread edematous areas and necrotic cells in the islets of Langerhans in the MTX. We found that necrotic cells and edematous areas were decreased in the TPM compared to the MTX. We found fewer β cells showing insulin positivity in the MTX compared to the controls ($p<0.05$). We observed an increase in insulin-positive β-cells in the TPM compared to the MTX ($p<0.05$). We observed a significant decrease in the number of cells showing glucagon positivity in the MTX compared to the controls ($p<0.05$). We observed an increased number of α cells showing glucagon positivity in the TPM compared to the MTX ($p<0.05$). We found that the HDS increased from 0(0-1) in the control group to 6(6-8) in the MTX ($p<0.05$). It was found as 2(2-3) in the TPM ($p<0.05$).
Conclusion: Our study shows that topiramate has a protective effect on pancreatic islet cells.

Keywords: Methotrexate, pancreatic injury, rat, topiramate

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INTRODUCTION

Topiramate (TPM) is an anti-epileptic drug indicated for the treatment of partial and generalized epileptic attacks in adults and children that possess different mechanisms than other antiepileptic drugs (1). TPM, which is accepted as an antiepileptic and neuroprotective agent, has found an area of use either as a single agent or in combination with other drugs in a multitude of conditions including TPM, migraine episodes, essential tremors, alcohol dependence, neuropathic pain, Lennox-Gastaut syndrome, bipolar disorder, and schizophrenia, as well as obesity in combination with phentermine. (2)

Although antiepileptic drugs are typically associated with weight gain, TPM differs from other antiepileptics as it is known to be associated with weight loss and a decrease in appetite (3). TPM was observed to exert an effect similar to that of antidiabetic drugs by achieving improved glycemic control in animal and human studies conducted to date (4-7). The molecular mechanism underlying the effects of TPM on glycemic control has not been clarified; however, this effect is independent of its effects that promote weight loss and decreased appetite (3).

Studies on the effects of TPM on glycemic control have proposed several mechanisms. One of these suggests that TPM reduces blood glucose levels by increasing glucose-stimulated insulin secretion (6). However, this hypoglycemic effect of TPM was not observed in healthy subjects and it did not alter glucose-stimulated insulin secretion in these individuals (8, 9). Therefore, the strongest mechanism that has been proposed is that the increase in insulin secretion might be linked to the protective effects of TPM on islet beta cell function (4, 8, 10). Published data suggest that the improvement of b-cell function in lipid and/or glucotoxicity-related models may explain the antidiabetic effects of TPM (4, 7, 8).

Studies have shown that TPM has neuroprotective and antiapoptotic effects (11). It has been suggested that the reduction of apoptotic activity might have favorable effects on pancreatic injury as well as on the central nervous system (12). Accordingly, TPM may be improving pancreatic tissue injury and beta cell function through its direct trophic effects in addition to its metabolic effects on beta cells. Almost all studies conducted on pancreatic islet beta cell function with TPM have included either obese or diabetic patients or animal models (4, 9, 13). It is known that TPM has no effect related to glucose regulation or pancreatic beta cell function in healthy individuals with normal weight (7, 8, 10). To our knowledge, no more than one study investigated the protective effects of TPM on pancreatic injury related to causes other than obesity and diabetes (12).

Methotrexate (MTX) is a chemotherapeutic drug that essentially takes effect by inhibiting the synthesis of thymidylate and the folic acid cycle and results in the impairment of nucleic acid synthesis, which leads to cell death (14). Toxicity studies on methotrexate, which is an antimetabolite used in the treatment of many cancers and autoimmune diseases, have mostly included the liver, kidneys, and the lung (15-19). However, MTX-based chemotherapy often results in various toxicities that require dose reduction or treatment cessation (16). There are very few studies in the literature showing that MTX may induce pancreatic toxicity.

Based on the neuroprotective and antiapoptotic effects of TPM, we hypothesized that it could have a protective effect on pancreatic injury and islet cells. Another aim of our study is to investigate the potential damage in endocrine pancreatic tissues that methotrexate exposure causes using histopathological and immunohistochemical methods.

MATERIALS AND METHODS

This study included 24 male, 3-4-month-old Sprague-Dawley rats weighing 300±30g. Subjects were obtained from the Animal Research Unit of Recep Tayyip Erdogan University. The study was approved by the local ethics committee for animal research at Recep Tayyip Erdogan University (ID24, Date: 30/06/2022). The study was conducted at Recep Tayyip Erdogan University Animal Research Unit between July 4-July 24, 2022. The subjects were kept under optimal conditions with constant temperature (21±2°C) and photoperiod (12:12 hour light-darkness cycle). All animals were allowed ad-libitum access to water and food. The sample size was calculated based on the method described by Charian et al. (22). With the use of a numerator software, the subjects were randomly assigned to one of three groups: control group (n=8), methotrexate group (n=8), and methotrexate + topiramate group (n=8).

Chemicals and Medications

Methotrexate “EBEWE (50 mg/5 ml Ebewe Pharma GmbH Nfg. KG Mondseestrasse 11 A–4866 Unterach, Austria). Topiramate (TOPAMAX 25 mg, Johnson and Johnson Medical Products Industry and Trade Ltd., Cilag AG - Schaffhausen/Switzerland). Ketamine HCL (Ketalar 500 mg, Pfizer Pharmaceuticals Ltd.

Co. Ortakoy, Istanbul, Turkey). Xylazine (Rompun 2%, Bayer, Turkey, Istanbul, Turkey).

Experimental Procedure

Rats in the control group were only given a single intraperitoneal (i.p.) dose of 1 mL of 0.9% physiological serum (Biofleks, Osel Pharmaceuticals Industry and Trade Inc., Beykoz, Istanbul, Turkey). The MTX group was given a single dose of 20mg/kg MTX (19, 23). The TPM treatment group was given a single dose of 100 mg/kg/day by oral gavage over 14 days; 7 days before and 7 days after the administration of MTX (24, 25). All subjects were euthanized with 50 mg/kg Ketamine HCL and 10 mg/kg Xylazine HCl seven days from the TPM application and 16 hours from the final dose.

Histopathological Analysis

Pancreatic tissue specimens excised from the rats were trimmed and fixed in 10% formalin for 48 hours. Following the fixation procedure, pancreas specimens were transferred to a tissue processing cassette (Isolab GmbH, Germany) and passed through ethanol (Merck GmbH, Germany) series of increasing concentration (50%, 70%, 80%, 90%, 96%, 100%, 100%) using a tissue processor (Shandon Citadel 2000, Thermo Sceintific Inc., Germany). In the next stage, pancreatic tissue specimens were kept in two series of xylol (Merck GmbH, Germany) and subjected to an embedding procedure with soft (Merck GmbH, Germany) and hard paraffin (Merck GmbH, Germany). Pancreatic tissue specimens that were kept overnight in paraffin were then embedded in tissue embedding cassettes (Isolab GmbH, Germany) using a paraffin embedding station (Leica Biosystems, EG1160, Germany). From the resulting paraffin blocks of pancreatic tissue, 4-5-micrometer sections were obtained using a rotary microtome (RM2525, Leica Biosystems, Germany). The resulting pancreatic tissue sections were stained with Harris hematoxylin (Merck GmbH, Germany) and Eosin G (Merck GmbH, Germany) using a staining device (Leica 5020ST, Leica Biosystems, Germany)

Immunohistochemical Analysis

2-3-micrometer sections obtained from the paraffin blocks (RM2525, Leica Biosystems, Germany) of pancreatic tissue were transferred onto positively charged slides. The primary insulin antibody kit (ab15147, Abcam, United Kingdom) was used to identify the insulin-secreting β cells in the islets of Langerhans and the primary glucagon antibody kit (ab92517, Abcam, United Kingdom) was used to identify the glucagon-secreting α-cells. Secondary antibody kits corresponding to the primary antibodies (Goat Anti-Rabbit IgG H&L (HRP), ab97051, Abcam, United Kingdom) were used. Primary and secondary antibody kits were incubated using an ICH/ISH staining device (Leica Bond Max, Leica Biosystems, Germany) according to the instructions manual of the manufacturer. Harris Hematoxylin (Merck GmbH, Germany) was used for counter-staining.

Semi-quantitative Analysis

A Histopathological Damage Score (HDS) was developed for the histopathological scoring of pancreatic tissue according to the study by Schmidt et al. (26) as shown in Table 1. Twenty randomly selected fields per preparation were scored by an experienced histopathologist. The histopathologist was blind to the study groups.

Immunopositive cells in the pancreatic tissues incubated with the primary antibodies were scored using immunohistochemical methods as shown in Table 2. Twenty randomly selected fields per preparation were scored by an experienced histopathologist. The histopathologist was blind to the

Table 1. The pancreatic Histopathological Damage Score (PHDS) was modified based on the Damage Score by Schmidt et al.

Score	Findings
Necrotic Cells	
0	≤5%
1	Between 6%-25%
2	Between 25%-50%
3	≥50%
Edematous Areas	
0	≤5%
1	Between 6%-25%
2	Between 25%-50%
3	≥50%
Cells with Pyknotic Nuclei	
0	≤%5
1	Between 6%-25%
2	Between 25%-50%
3	≥50%

Table 2. Immunohistochemical Positivity Scoring

Score	Findings
0	≤5%
1	Between 6%-25%
2	Between 25%-50%
3	≥50%

Table 3. Pancreatic Histopathological Damage Score (PHDS) Results (median-(25%-75% values)).

Gruplar	Necrotic Cells	Edematous Areas	Cells with Pyknotic Nuclei	PHDS
Control	0(0-0)	0(0-0)	0(0-0)	0(0-1)
MTX	2(2-2)*	2(2-2)*	2(2-2)*	6(6-7)*
MTX+TPM	1(0-1)**,***	0(0-1)***	1(1-1)**,***	2(1-3)**,***

* p<0.001; Between the control group and the MTX group,
** p<0.001; Between the control group and the MTX+TPM group,
***p<0.001; Between the MTX group and the MTX+TPM group,
Kruskal Wallis/Mann Whitney U test

study groups.

Statistical Analysis

Data obtained using histopathological and immunohistochemical methods were subjected to Shapiro-Wilk, Q-Q plot, and Skewness-Kurtosis analyses using the SPSS 20 (IBM Corp., USA) computer software. For non-parametric data; median (25% and 75% interquartile range) values were calculated. The differences between the groups were subjected to the Kruskal-Wallis test and then to the Bonferroni-corrected Mann-Whitney U test. P <0.05 was accepted as statistically significant.

RESULTS

Histopathological Analysis

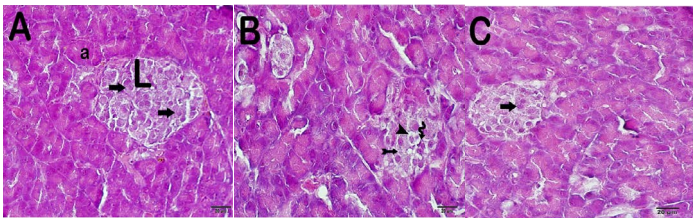

Figure 1. Light microscopic image of H+E-stained pancreatic tissue.
Langerhans Islets (L), Acinus (a)
A(x20) Control Group: Pancreatic islet of Langerhans (L) showing normal cells (arrow). B (x20) MTX treatment group: Pancreatic islet of Langerhans (L) showing extensive loss of cytoplasm accompanied by necrotic cells (tailed arrow) with vacuolizations. In addition, numerous necrotic cells are observed to form edematous regions in the islets of Langerhans (spiral arrow). Also, pancreatic cells with pyknotic nuclei can be observed at places (arrowhead). C(x20) Topiramate Treatment Group: Islets of Langerhans (L) showing extensive typical cells (arrow) with fewer necrotic cells and decreased edematous areas (arrowhead).

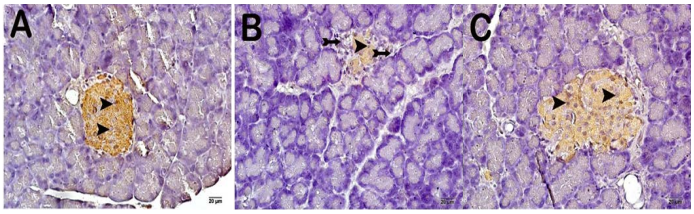

Figure 2. Light microscopic image of pancreatic tissue incubated with insulin primary antibody.
A(x20) Control Group: β cells (arrowhead) showing extensive insulin positivity in normal islets of Langerhans B(x40) MTX Group: Fewer insulin-positive β cells (tailed arrow) observed in degenerative islets of Langerhans. C(x40) MTX+TPM Treatment Group: More β cells showing insulin positivity (tailed arrow) in typical islets of Langerhans.

On examination of H+E stained sections under a light microscope, we observed extensive edematous areas and necrotic cells in the islets of Langerhans in the MTX treatment group (Figure 1a-b). In contrast, we determined a decrease in necrotic cells

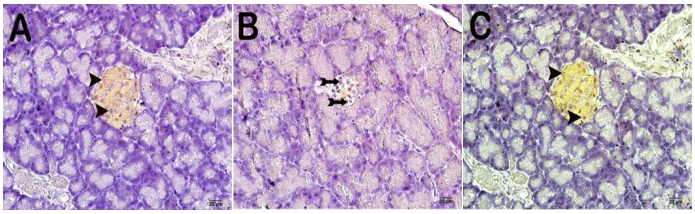

Figure 3. Light microscopic image of pancreatic tissue incubated with glucagon primary antibody.
A(x20) Control Group: Normal α cells (arrowhead) showing extensive insulin positivity in the islets of Langerhans B(x40) MTX Group: Atypical islets of Langerhans showing reduced insulinp ositivity in α cells (tailed arrow). C(x40) MTX+TPM Treatment Group: An increased amount of typical α cells (tailed arrow) showing insulin positivity in the islets of Langerhans

Table 4. Semi-quantitative analysis (median (values between 25%-75% quartiles)

Groups	Anti-Insulin Positivity Score	Anti-Glucagon Positivity Score
Control	3(2-3)	2(2-2.5)
MTX	0.5(0-1)*	0.5(0-1)*
MTX+TPM	2(2-2)**	1(1-2)**

* p<0.001; Between the control group and the MTX group,
**p<0.001; Between the MTX group and the MTX+TPM group,
Kruskal Wallis/Mann Whitney U test

and edematous areas in the TPM treatment group compared to the MTX treatment group (Figure 2b-c).

Immunohistochemical Analysis

On examination of the sections of pancreatic tissue incubated with insulin primary antibody under a light microscope, we determined fewer β-cells showing insulin positivity in the MTX group compared to the control group (Figure 2a-b, Table 4, p<0.001). On the other hand, we observed a significant increase in β-cells showing insulin positivity in sections of pancreatic tissue belonging to the TPM treatment group compared to the MTX group (Figure 2b-c, Tablo 4, p<0.001).

On examination of the sections of pancreatic tissue incubated with glucagon primary antibody under a light microscope; we determined significantly fewer cells showing glucagon positivity in the MTX group compared to the control group (Figure 3a-b, Table 4, p<0.05). In contrast, in the TPM treatment group, we observed an increase in α-cells showing glucagon positivity compared to the MTX group (Figure 3b-c, Table 4, p<0.05).

Semi-quantitative Analysis

The HDS score calculated concerning edematous areas, necrotic cells, and cells with pyknotic nuclei were determined as 0 (0-1) in the control group and as 6(6-7) in the MTX group (Figure 1a-b, Table 3; p<0.001). However, the HDS score calculated as 6 (6-7) for the MTX group was found as 2(1-3) in the TPM treatment group (Figure 1b-c, Table 3, p<0.001).

DISCUSSION

In this study, the effects of MTX on pancreatic tissue and the effects of TPM on rats treated with MTX were evaluated histopathologically and immunohistochemically. Methotrexate treatment-induced damage in pancreatic islets of Langerhans cells. According to the results of our literature review, there are very few studies on MTX-induced pancreatic toxicity and these have shown high-dose MTX to result in atypical islets of Langerhans, mild edema, necrotic cells in the islets of Langerhans and acinar cells, and inflammatory infiltration (20). Similarly, we observed extensive edematous areas and necrotic cells in the islets of Langerhans after MTX treatment in the present study. Again, the immunohistochemical analysis performed in the present study determined a decrease in the number of cells showing insulin and glucagon positivity, in line with the literature. There are studies in the literature that report MTX-induced liver, kidney, and lung toxicity and show MTX-induced apoptosis and inflammation in liver and kidney tissues (16, 21). In a case report on MTX-induced pulmonary toxicity, reactive epithelial hyperplasia, as well as focal histiocyte clusters in the alveoli and chronic lymphocytic interstitial inflammation, were found on lung biopsy (15, 23). MTX causes impairment of nucleic acid synthesis, and thus, cell apoptosis by inhibiting thymidylate synthesis and the folic acid cycle (14). This effect is more pronounced in malignant cells. The mechanism by which MTX induces pancreatic injury is likely linked to apoptosis. Our study is one of the very rare studies that have demonstrated pancreatic injury induced by MTX.

Consistent with the literature, our study observed a protective effect of TPM on the islets of Langerhans cells. The recognition that TPM causes decreased appetite, weight loss, and, in diabetic patients, reduced blood glucose in contrast with other antiepileptics has stimulated a great amount of research on its anti-obesity and antidiabetic aspects in addition to its antiepileptic effects. Many studies have been conducted to determine whether the effect of TPM that decreases blood sugar is through its effects that increase insulin sensitivity due to weight loss or through its effects that replace beta cells (5, 6, 8). The few studies showing that TPM reduces blood glucose due to elevated insulin sensitivity in peripheral tissues as a result of weight loss and higher energy consumption were not corroborated by further studies and it was understood that the blood glucose reduction effect was independent of weight

loss and energy consumption (27). Furthermore, the absence of this blood glucose reduction effect in healthy individuals despite its presence in diabetics guided research towards the idea that TPM improves impaired beta cell function (8, 9).

Pancreatic islet cells are quite sensitive to lipotoxicity (10). The exposure of beta cells to lipotoxicity initiates the metabolic processes that lead to lipid accumulation, mitochondrial dysfunction, and ultimately, reduced insulin secretion (10). In addition to being proven by the partial loss of mitochondrial resting membrane potential and reduced hyperpolarization in response to glucose, the lipotoxic effects have also been linked to impaired mitochondrial function (8). TPM increases the beta-oxidation rate by elevating the expression of PPAR alpha and CPT-1, which is a mitochondrial fatty acid carrier (8). The most important mechanism is presumed to be the direct antiapoptotic effect TPM exerts on b-cells by offering protection against lipotoxicity (10).

Certain studies involving animal models of obesity and diabetes have shown that TPM improves insulin sensitivity independently of weight loss (6). The associated mechanisms are uncertain; however, studies have speculated that adipose tissue might be a target. TPM was shown to improve the effects of insulin and the transportation of glucose in fat cells obtained from obese and insulin-resistant rodents, as well as to elevate adiponectin secretion (5, 9). Despite the favorable effects of TPM on weight and glycemia, human studies on type II DM did not yield any evidence suggesting that it alters insulin sensitivity (5, 9).

In addition to hormones, pancreatic islet cells also secrete neurotransmitters. It is known that pancreatic islet cells secrete glutamate decarboxylase and GAMA transaminase (28). GABA, which is released from synaptic vesicles on beta cells, binds to the ionotropic GABA A receptors found on a-cells and leads to the inhibition of glucagon secretion, while it also stimulates insulin secretion by binding to the GABA B receptors found on beta cells (28, 29). Studies are showing that TPM inhibits glucagon secretion through the reinforcement of GABA activity by increasing GABA activity in some GABA A receptor subtypes (1).

Islet cells express ionotropic (iGluRs) and metabotropic (mGluRs) glutamate receptors (29). From ionotropic glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) modulates the pancreatic secretion of insulin and glucagon ((2). The activation of ionotropic glutamate receptors (iGluRs) leads to an increase in the concentration of cytosolic Ca^{+2} ($[Ca^{+2}]i$). The constant increase in ($[Ca^{+2}]i$) due to the overstimulation of these receptors may result in impaired cell function, and thus, cell apoptosis (30). Therefore, TPM blocks kainate and AMPA receptors from glutamate receptor subtypes (13).

Carbonic anhydrase (CA) VA and CA VB are known to be involved in certain metabolic processes including urogenesis, glucogenesis, and lipogenesis (31). TPM was shown to inhibit many CA isoforms including mitochondrial CA VA and CA VB (1). All of these results support that TPM inhibits mitochondrial CAs, and therefore, de novo lipogenesis (31).

To our knowledge, there are no studies in the literature that have suggested any negative effects on the pancreas by TPM. However, studies that have shown protective and anti-apoptotic effects on neuronal cells led us to think that TPM could exert a protective effect on the pancreas as well. In their experimental study, Niebauer and Gruenthal showed reduced neuronal degeneration in the hippocampal area of the brain after status epilepticus (32). Similarly, Kurul et al. (33) showed by inducing experimental hyperoxic brain injury that TPM yielded a significant decrease in cell death in the hippocampal region. The study by Park et al. (30) has also shown that TPM produces a decrease in cell death-related seizures. However, it has not been elucidated whether this protective effect is caused by the metabolic effects or the trophic effects of TPM or by a combination of these two effects, as well as which effect is more dominant. Apart from its direct trophic effects on islet cells, topiramate may indirectly improve beta cell function through metabolic effects.

In this study, we aimed to investigate whether TPM exerts a protective effect on the islets of Langerhans cells, by inducing pancreatic toxicity through the treatment of rats with MTX. Certain limitations of this study should be considered. Firstly, this is a pilot study conducted by inducing pancreatic injury using MTX. Therefore, there are not enough studies in the literature to perform a comparison. We did not determine the levels of insulin and glucose in this study. The molecular mechanisms of the effects of MTX and TPM on the pancreas need to be supported by future studies.

CONCLUSION

In summary, this study shows that MTX induces cellular damage in pancreatic tissues and that TPM

has a protective effect on pancreatic islet cells. More experimental and clinical studies are needed to investigate the mechanisms underlying the protective effects of various doses of TPM in pancreatic beta cell damage in both type 1 and type 2 diabetes. When TPM therapy is planned for diabetic epilepsy patients on insulin or insulin secretagogues, hypoglycemia should be given consideration, and perhaps, the option of reducing the doses of insulin should be preferred.

Conflict of interest: *Authors declare that there is no conflict of interest between the authors of the article.*

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