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Histological assessment of the hippocampus in type 2 diabetic rats: Neuroprotective roles of exenatide, empagliflozin, and quercetin

Evaluación histológica del hipocampo en ratas con diabetes tipo 2: funciones neuroprotectoras de la exenatida, la empagliflozina y la quercetina

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ABSTRACT

This study aimed to evaluate the histological changes in hippocampal tissue following exenatide, empagliflozin, and quercetin monotherapy, as well as combined treatment, in rats with type 2 diabetes. The rats were divided into 7 groups: group 1 (non–diabetic control), group 2 (diabetic control), group 3 (diabetic + sham), group 4 (diabetic + exenatide, 10 $\mu g \cdot kg^{-1}$), group 5 (diabetic + empagliflozin, 50 $mg \cdot kg^{-1}$), group 6 (diabetic + quercetin, 50 $mg \cdot kg^{-1}$), and group 7 (diabetic + combination treatment). The study lasted for 8 weeks. At the end of the study, brain tissues of the rats were collected and fixed in 10% buffered formaldehyde. After fixation, routine tissue processing was performed, and paraffin blocks were created. May–Grünwald Giemsa staining and Argyrophilic nucleolar organizer region staining were applied to the paraffin sections. The CA1, CA3, and dentate gyrus regions of the hippocampus were evaluated. The study revealed an increase in the number of shrunken, dark neurons with pyknotic nuclei in diabetic rats, while a decrease in the number of healthy neurons was observed. Cell proliferation was assessed using Argyrophilic nucleolar organizer region staining, which showed that diabetes causes a decrease in cell proliferation. Exenatide, empagliflozin, and quercetin ameliorated the diabetes–induced decrease in the number of healthy neurons, and combination therapy yielded better results than monotherapy.

Key words: Diabetes; empagliflozin; exenatide; hippocampus; quercetin

RESUMEN

Este estudio tuvo como objetivo evaluar los cambios histológicos en el tejido hipocampal después de la monoterapia con exenatida, empagliflozina y quercetina, así como el tratamiento combinado, en ratas con diabetes tipo 2. Las ratas se dividieron en 7 grupos: grupo 1 (control no diabético), grupo 2 (control diabético), grupo 3 (diabético + placebo), grupo 4 (diabético + exenatida, 10 $\mu g \cdot kg^{-1}$), grupo 5 (diabético + empagliflozina, 50 $mg \cdot kg^{-1}$), grupo 6 (diabético + quercetina, 50 $mg \cdot kg^{-1}$) y grupo 7 (diabético + tratamiento combinado). El estudio duró 8 semanas. Al final del estudio, se recolectaron tejidos cerebrales de las ratas y se fijaron en formaldehído bufferado al 10 %. Después de la fijación, se realizó el procesamiento rutinario del tejido y se crearon bloques de parafina. Se aplicaron tinciones de May–Grünwald Giemsa y Región organizadora nucleolar argirófila a las secciones de parafina. Se evaluaron las regiones CA1, CA3 y del giro dentado del hipocampo. El estudio reveló un aumento en el número de neuronas encogidas y oscuras con núcleos picnóticos en ratas diabéticas, mientras que se observó una disminución en el número de neuronas sanas. La proliferación celular se evaluó mediante tinción con Región organizadora nucleolar argirófila, que mostró que la diabetes causa una disminución en la proliferación celular. La exenatida, la empagliflozina y la quercetina mejoraron la disminución del número de neuronas sanas inducida por la diabetes, y la terapia combinada produjo mejores resultados que la monoterapia.

Palabras clave: Diabetes; empagliflozina; exenatida; hipocampo; quercetina

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INTRODUCTION

Diabetes is a common and serious metabolic disorder. Although there are various types of diabetes, the most well–known are type 1 and type 2 diabetes [1]. Type 2 diabetes accounts for approximately 90% of reported cases worldwide. Type 2 diabetes occurs due to decreased insulin sensitivity in peripheral tissues. As the disease progresses, insufficient insulin production occurs as a result of dysfunction of the pancreatic beta cells [2]. Studies have reported that insulin receptors are found in the brain and that it is sensitive to insulin. One of the areas where these receptors are found is the hippocampus [3, 4, 5].

The hippocampus is part of the limbic system and is involved in learning, memory consolidation, and emotional regulation. In addition to these functions, it also contributes to the coordination of several cognitive processes [6]. Compared with many other brain regions, the hippocampus shows increased sensitivity to metabolic disturbances, including those observed in diabetes [1]. In this context, diabetes has been associated with various neurological complications, particularly impairments in cognitive function and the development of depressive symptoms [2].

Experimental studies using streptozotocin–induced diabetic models have described notable structural changes in the hippocampus, including neuronal loss and an increased number of darkly stained neurons [7, 8]. Such alterations are widely attributed to oxidative stress, which plays a major role in the development of diabetes–related complications [9]. Under conditions of chronic hyperglycemia, increased oxidative stress contributes to neuroinflammatory activity, disrupts mitochondrial function, and leads to neuronal damage that supports the progression of neurodegenerative changes in the brain [10, 11, 12].

When lifestyle–based measures such as dietary regulation and physical activity are insufficient to control blood glucose levels, pharmacological approaches are commonly required in diabetes management. Exenatide and empagliflozin are frequently discussed among current treatment options because of their distinct mechanisms of action. In addition to conventional therapies, plant–derived compounds, including quercetin, have also been examined as supportive agents. Exenatide acts as a glucagon–like peptide–1 (GLP–1) receptor agonist, increasing glucose–dependent insulin secretion, and its effects have been associated with potential neuroprotective properties [13, 14].

Empagliflozin is used in the treatment of type 2 diabetes as a member of the sodium–glucose cotransporter–2 (SGLT–2) inhibitor class. Its glucose–lowering effect results from increased urinary glucose excretion via the kidneys, which contributes to overall glycemic control. Available clinical evidence indicates that empagliflozin is generally well tolerated and effective in maintaining glucose balance in patients with type 2 diabetes [15, 16].

Quercetin is a flavonoid naturally found in many foods such as apples, various fruits, onions, tea, red wine, nuts, and many vegetables. It is of therapeutic interest due to its versatile pharmacological properties. In addition to its potent antioxidant effect, quercetin is reported to have a broad biological effect profile, including anti–inflammatory, anti–apoptotic, antimicrobial, antiviral, anti–ulcer, anti–cancer, hepatoprotective, antihypertensive, lipid–lowering, and neuroprotective effects [17, 18, 19, 20].

This study aimed to examine the potential neurotoxic impact of diabetes on the hippocampus and to evaluate the effects either protective or detrimental of exenatide, empagliflozin, and quercetin on this brain region. Furthermore, the research sought to determine whether administering these agents to diabetic rats (*Rattus norvegicus*) could mitigate or prevent the adverse effects of diabetes on hippocampal structure and function.

MATERIAL AND METHODS

This study was approved by the Selçuk University Veterinary Faculty Experimental Animal Production and Research Center Ethics Committee with the decision dated 06.03.2025 and numbered 2025/36. The hippocampal tissue samples used in this study were obtained from the study entitled “Investigation of the Antidiabetic Effects of Exenatide, Empagliflozin, and Quercetin in Type 2 Diabetic Rats”.

In animals with statistically comparable lipid profiles ($P>0.05$), an experimental type 2 diabetes model was established according to the protocol of Dik *et al.* [21]. In this approach, animals were first maintained for two weeks on a high–fat diet formulated to provide approximately 58% of the metabolizable energy from animal fat. Thereafter, streptozotosin was freshly prepared in citrate buffer (pH 4.5) on ice, and the purity and supplier of the compound were specified. Following a 6–8 hour (h) fasting period, a single low dose of streptozotocin (35 $mg \cdot kg^{-1}$, SC) was administered subcutaneously.

Diabetes was confirmed on the fifth day (d) after injection by tail vein blood sampling using a glucometer, and rats with blood glucose levels ≥ 300 $mg \cdot dL^{-1}$ consistent with values reported in the literature were classified as diabetic. Once hyperglycemia was confirmed, the diabetic animals continued on the high–fat diet for an additional eight weeks to promote the progression of the metabolic disorder.

The high–fat diet was formulated to contain 3.0% vegetable oil, 37.0% animal fat, 30.5% yellow corn, 20.0% dried casein, 4.5% soybean meal (48% CP), 1.7% dicalcium phosphate, 0.2% DL–methionine, 1.6% limestone, 0.5% sodium chloride, and 1.0% vitamin–mineral premix.

This experimental study was carried out on forty–two male Wistar Albino rats, aged between 8 and 12 weeks. Throughout the research period, the animals were maintained under controlled environmental conditions, including a 12–h light/dark cycle, a constant room temperature of 22 ± 2°C, and relative humidity maintained at 55 ± 5%. The animals were randomly divided into seven groups as follows: group 1 (non–diabetic control), group 2 (diabetic control), group 3 (diabetic + sham), group 4 (diabetic + exenatide), group 5 (diabetic+empagliflozin), group 6 (diabetic+quercetin), and group 7 (diabetic+combination treatment).

Group 1: Rats in the control group were provided with standard rodent chow and had free access to water throughout the experiment.

Group 2: Serving as the diabetic control group, was fed a high–fat diet for eight consecutive weeks following the induction of diabetes, with no additional treatment applied.

Group 3: For eight weeks, rats received daily oral administrations of hydroxyethylcellulose (0.5 mL·kg^{-1}) and sunflower oil (0.5 mL·kg^{-1}), along with subcutaneous (sc) injections of physiological saline (0.1 mL·kg^{-1}) as a sham treatment.

Group 4: This group was treated with exenatide at a dosage of 10 μg·kg^{-1} via sc injection once daily for eight weeks.

Group 5: Rats in this group were given empagliflozin (50 mg·kg^{-1}·d^{-1}) by oral gavage, prepared in hydroxyethylcellulose, for eight consecutive weeks.

Group 6: Quercetin was administered orally at a dose of 50 mg·kg^{-1}·d^{-1}, dissolved in sunflower oil, for a period of eight weeks.

Group 7: For eight weeks, this group received a combination therapy consisting of exenatide (10 μg·kg^{-1}·d^{-1}, sc), empagliflozin (50 mg·kg^{-1}·d^{-1}, po), and quercetin (50 mg·kg^{-1}·d^{-1}, po).

At the end of the 8–week experimental period, the animals were anesthetized via intraperitoneal injection with Thiopental sodium at a dose of 40 mg·kg^{-1} (Pental Sodyum, I.E.Ulugay, Türkiye). Afterwards, the confirmation of type 2 diabetes should be supported by indices such as Homeostatic Model Assessment for Insulin Resistance (HOMA–IR), insulin measurements, or at least documentation of a stable hyperglycemic profile in the presence of circulating insulin. This validation has been demonstrated in detail by Korkmaz and Dik [16].

The brain tissues were removed from the rats euthanized under anesthesia and fixed in 10% buffered formaldehyde for 10 d. After the fixation process, routine tissue tracking was performed and paraffin blocks were created. Paraffin blocks were trimmed, and 4–6 μm thick sections were cut from the hippocampal region using a microtome (Leica, RM2125RT, Germany). May–Grünwald Giemsa staining and AgNor staining were performed on the sections taken. In the sections where May–Grünwald Giemsa staining was performed, neuron counts were performed in 3 different areas in the CA–1, CA–3 and Dentate gyrus regions of the hippocampus at 100× magnification. Measurements were performed by counting pyramidal neurons in an area of 15000 μm^2 (0.015 mm^2).

After the AgNor staining process, 25 pyramidal neurons in the CA1, CA3 regions and 25 granular neurons in the Dentate gyrus region of the hippocampus were evaluated in each preparation at X100 magnification. In each cell, measurements of the nuclear area, Argyrophilic nucleolar organizer region (AgNOR) count, and AgNOR area were carried out, and the relative AgNOR area (%) was calculated. The samples were examined using a Leica DM2500 light microscope (Leica Microsystems, Switzerland) equipped with a DFC–320 camera. Measurements were conducted with the LAS image analysis software, and images of selected regions were captured for documentation.

Statistical analysis

The collected data were analyzed statistically using analysis of variance (ANOVA), followed by Duncan's post hoc test to determine differences between the groups. All statistical analyses were performed using IBM SPSS software (version 22.0).

RESULTS AND DISCUSSION

Diabetes mellitus is a chronic metabolic disorder characterized by persistent hyperglycemia, which primarily arises from insufficient insulin secretion and/or impaired insulin action. Type 2 diabetes is distinguished by the coexistence of reduced insulin sensitivity in peripheral tissues and a relative deficiency in insulin production. In the present study, type 2 diabetes was experimentally induced using streptozotocin, a compound known for its cytotoxic effects on pancreatic β–cells, thereby replicating key aspects of the disease's pathophysiology [22, 23].

Numerous studies have demonstrated that insulin receptors are present in different regions of the brain, such as the hypothalamus, hippocampus, cerebral cortex, and thalamus, and that these regions are affected by insulin [24, 25, 26]. One of these regions, the hippocampus, is prominent in its function related to memory. The hippocampus plays a role in the conversion of short–term memory into long–term memory. Additionally, neurogenesis has been reported to occur in the dentate gyrus region [27].

In this study, the CA1, CA3, and dentate gyrus regions of the hippocampus were histopathologically evaluated, and histomorphometric measurements were performed in these regions. In the histopathological evaluation performed in Group 1, it was determined that the pyramidal neurons located in the CA1 region consisted of small, round–shaped, euchromatic nuclei and tightly packed healthy neurons. Pyramidal neurons in the CA3 region were observed to be larger and sparsely distributed. In the histopathological evaluation performed in Group 2, darkly stained, shrunken apoptotic neurons were observed in addition to healthy neurons. The histomorphometric measurements revealed a statistically significant decrease in the number of healthy neurons compared to Group 1.

The histopathological and histomorphometric evaluations performed in Group 3 showed similar results to those in Group 2. Both healthy neurons and apoptotic neurons were identified in the treatment groups. The histomorphometric measurements revealed an increase in the number of healthy neurons in the treatment groups compared to Group 2. The most pronounced results were observed in Group 7, followed by Groups 4, 5, and 6, respectively. Detailed measurement results and related images are presented in TABLE I and FIG. 1.

Histopathological evaluation of the dentate gyrus revealed numerous round, euchromatic healthy neurons in the stratum granulosum layer of group 1. In Group 2, darkly stained, shrunken, and degenerated apoptotic neurons were identified alongside healthy neurons. Group 3 showed a morphology similar to Group 2. The presence of healthy neurons and apoptotic neurons was determined in the treatment groups (Groups 4, 5, 6, and 7). Histomorphometric evaluations revealed that diabetes caused a statistically significant decrease in the number of healthy neurons. An increase in the number of healthy neurons was observed in the treatment groups, with the most pronounced result seen in group 7. Detailed measurement data and figures are presented in TABLE I and FIG. 1.

In diabetes, impairments in glucose oxidation, non–enzymatic glycation of proteins, and oxidative degradation of glycated proteins lead to increased free radical production [23, 28].

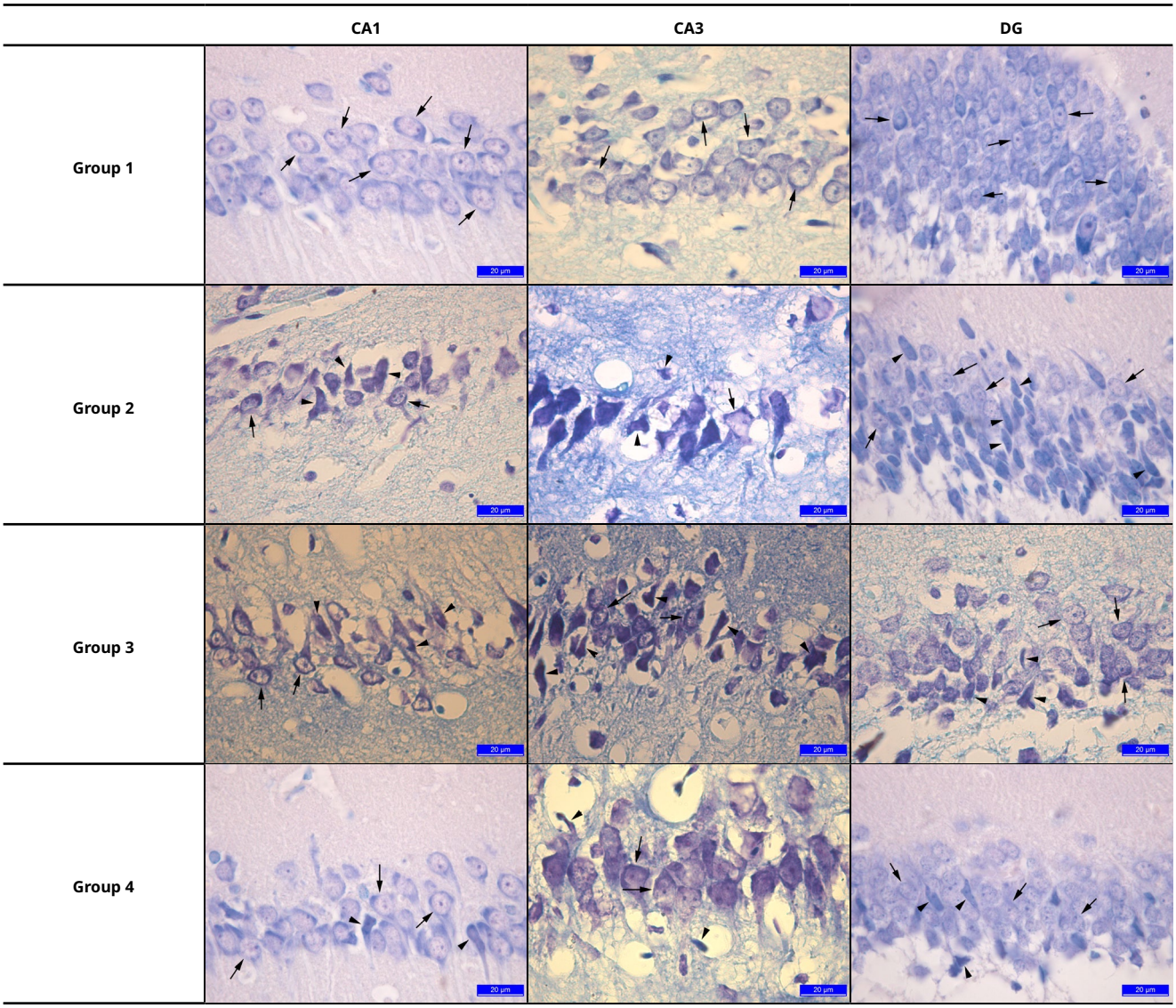
TABLE I
Quantitative assessment of neuron populations in hippocampal subfields: CA1, CA3, and dentate gyrus

	CA1	CA3	Dentate Gyrus
Group 1	37.94 ± 0.20^{a}	17.44 ± 0.46^{a}	75.16 ± 0.95^{ab}
Group 2	27.88 ± 0.68^{b}	11.88 ± 0.90^{b}	59.27 ± 1.39^{c}
Group 3	27.44 ± 0.68^{b}	12.38 ± 0.59^{b}	59.33 ± 0.96^{c}
Group 4	35.16 ± 0.81^{cd}	16.72 ± 0.35^{ad}	73.88 ± 1.53^{ab}
Group 5	34.66 ± 0.66^{c}	15.61 ± 0.47^{cd}	72.61 ± 0.72^{ab}
Group 6	32.66 ± 0.38^{e}	14.27 ± 0.36^{c}	71.55 ± 0.82^{b}
Group 7	36.55 ± 0.69^{ad}	17.27 ± 0.49^{a}	75.66 ± 2.04^{a}

Group 1: Control group, Group 2: Diabetic group, Group 3: Diabet + sham group, Group 4: Diabet + exenatide, Group 5: Diabet + empagliflozin, Group 6: Diabet + Quercetin, Group 7: Diabet + combination therapy group. Neuronal counts are expressed as mean ± SEM. Superscript letters ($^{a, b, c, d, e}$) indicate statistically significant differences between groups (P<0.05)

Therefore, oxidative stress is considered a fundamental mechanism in the onset and progression of metabolic diseases such as diabetes mellitus; it is also associated with the development of complications accompanying diabetes, particularly peripheral neuropathy, as well as neurodegenerative disorders such as sporadic Alzheimer's disease [9, 29]. Elevated free radical levels cause serious cellular damage to proteins, cell membrane lipids, and nucleic acids, ultimately leading to cell death [28, 30, 31].

In recent years, numerous studies have investigated the effects of diabetes on the hippocampus [32, 33, 34]. Studies conducted on diabetic rats have reported distinct histopathological changes, such as the presence of darkly stained, shrunken neurons in the CA1, CA3, and dentate gyrus regions [35]. In addition, studies have reported a decrease in the number of healthy neurons and an increase in the number of apoptotic neurons.



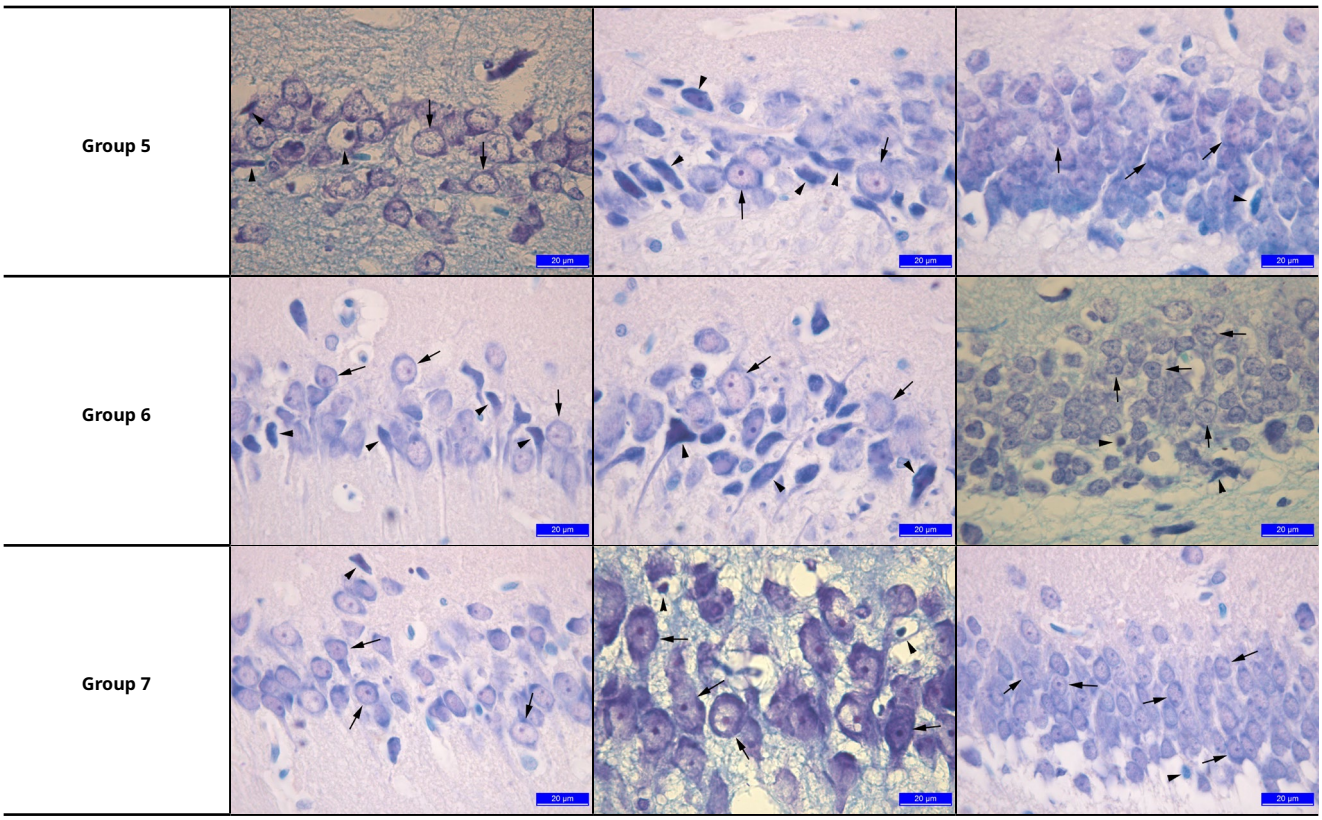


FIGURE 1. Group 1: Control group, Group 2: Diabetic group, Group 3: Diabet + sham group, Group 4: Diabet + exenatide, Group 5: Diabet + empagliflozin, Group 6: Diabet + Quercetin, Group 7: Diabet + combination thereapy group. Histological evaluation of neuronal morphology in the CA1, CA3, and dentate gyrus regions of the hippocampus following treatment with exenatide, empagliflozin, quercetin, and their combination in diabetic rats. Arrows indicate normal–appearing neurons; arrowheads mark darkly stained, degenerated neurons. May–Grünwald Giemsa staining. Scale bar: 20 µm. Magnification: 100×

One study found that 8 weeks of diabetes caused a significant increase in the number of apoptotic cells in the hippocampus [36]. Another study showed that 8 weeks of diabetes caused an increase in malondialdehyde (MDA) levels, superoxide dismutase (SOD) and catalase (CAT) activities, and the number of apoptotic neurons [37].

Similarly, another study also reported that diabetes caused an increase in the number of apoptotic neurons [38]. Studies have shown that diabetes activates apoptotic processes and causes neurodegeneration, leading to a decrease in the number of healthy neurons [39, 40, 41, 42]. Based on these findings, it is generally accepted that the oxidative stress caused by diabetes triggers apoptosis in the hippocampus and leads to neuron loss. Similar to the literature data, this study also found a significant decrease in the number of healthy neurons in diabetic rats. The results obtained support the idea that diabetes causes neurodegenerative changes in the hippocampus.

Numerous studies have shown that new neurons continue to form throughout life in the adult mammalian brain, including in humans [43]. The dentate gyrus region of the hippocampus, one of the main centers of adult neurogenesis, is closely related to cognitive functions such as learning and memory. It has been reported that neuronal loss in the dentate gyrus negatively affects the neurogenesis process, which may contribute to the development of Alzheimer's disease as well as memory and learning impairments [44].

Increasing evidence shows a strong relationship between diabetes and cognitive impairment and reduced neurogenesis [45, 46]. Indeed, Jackson–Guilford *et al.* [46] reported that diabetes reduces cellular proliferation in the dentate gyrus and that this is associated with memory impairments.

Similarly, Yi *et al.* [47] demonstrated that both cell proliferation and neuronal differentiation were significantly suppressed in the dentate gyrus in a streptozotocin–induced diabetes model. In this study, granular neurons in the dentate gyrus region were quantitatively assessed, and a significant decrease in the number of these cells was found to be associated with diabetes. Furthermore, findings of neuronal degeneration were also observed in the same region. The results obtained are consistent with previous findings reported in the literature.

Findings related to AgNOR parameters are summarized in TABLE II. In the CA1 and CA3 regions of the hippocampus, the number of AgNORs, AgNOR area, nucleus area, and relative AgNOR area in Group 2 were found to be statistically significantly reduced compared to Group 1 (P<0.05).

Based on the evaluations, the most positive results were obtained in Group 7, followed by Groups 4, 5, and 6, respectively. A similar trend was observed in the dentate gyrus region. In Group 2, a significant decrease in AgNOR count, AgNOR area, and relative AgNOR area was determined compared to Group 1 (P<0,05), while

TABLE II
Assessment of AgNOR parameters in hippocampal neurons: CA1, CA3, and Dentate Gyrus subregions

		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
CA 1	AgNOR Number	2.95 ± 0.19^{ab}	1.98 ± 0.12^{c}	2.03 ± 0.11^{c}	3.21 ± 0.13^{a}	3.06 ± 0.17^{ab}	2.68 ± 0.06^{b}	3.33 ± 0.13^{a}
	AgNOR Area (μm²)	6.25 ± 0.13^{a}	4.35 ± 0.18^{b}	4.31 ± 0.16^{b}	6.01 ± 0.28^{ad}	5.48 ± 0.17^{cd}	5.26 ± 0.15^{c}	6.25 ± 0.21^{a}
	Nucleus Area (μm²)	67.70 ± 1.44^{a}	59.23 ± 0.76^{b}	59.30 ± 0.97^{b}	66.16 ± 2.59^{a}	64.13 ± 1.87^{ab}	59.95 ± 1.77^{b}	67.33 ± 1.83^{a}
	Relative (%) AgNOR Area	9.24 ± 0.23^{a}	7.35 ± 0.36^{b}	7.27 ± 0.22^{b}	9.24 ± 0.77^{a}	8.60 ± 0.43^{a}	8.81±0.32^{a}	9.30 ± 0.30^{a}
CA 3	AgNOR Number	3.51 ± 0.14^{ab}	2.15 ± 0.19^{c}	2.08 ± 0.23^{c}	3.33 ± 0.20^{ab}	3.16 ± 0.12^{ab}	2.93 ± 0.24^{b}	3.5 5± 0.11^{a}
	AgNOR Area (μm²)	8.40 ± 0.17^{a}	6.91 ± 0.20^{c}	6.65 ± 0.29^{c}	8.25 ± 0.26^{a}	7.80 ± 0.20^{ab}	7.35 ± 0.26^{bc}	8.30 ± 0.30^{a}
	Nucleus Area (μm²)	83.16 ± 0.85^{a}	76.50 ± 1.41^{c}	77.11 ± 1.09^{c}	8068 ± 0.43^{ab}	79.61 ± 1.04^{abc}	78.58 ± 1.47^{bc}	82.45 ± 1.22^{a}
	Relative (%) AgNOR Area	10.12 ± 0.21^{a}	9.05 ± 0.28^{bc}	8.61 ± 0.33^{c}	10.22 ± 0.29^{a}	9.81 ± 0.36^{ab}	9.35 ± 0.31^{abc}	10.05 ± 0.27^{a}
Dentat Gyrus	AgNOR Number	4.68 ± 0.20^{a}	2.86 ± 0.09^{b}	2.93 ± 0.17^{b}	4.06 ± 0.13^{c}	3.73 ± 0.16^{cd}	3.26 ± 0.13^{bd}	4.23 ± 0.26^{ac}
	AgNOR Area (μm²)	5.48 ± 0.33^{a}	3.60 ± 0.27^{b}	3.58 ± 0.16^{b}	4.63 ± 0.15^{cd}	4.40 ± 0.18^{c}	4.13 ± 0.18^{bc}	5.18 ± 0.14^{ad}
	Nucleus Area (μm²)	40.20 ± 0.92	39.26 ± 1.01	37.18 ± 1.78	40.25 ± 1.80	38.91 ± 2.13	40.40 ± 2.58	39.93 ± 1.76
	Relative (%) AgNOR Area	13.73 ± 1.09^{a}	9.22 ± 1.96^{b}	9.81 ± 0.77^{b}	11.65 ± 0.73^{ab}	11.50 ± 0.86^{ab}	10.40 ± 0.69^{b}	13.15 ± 0.89^{a}

Group 1: Control group, Group 2: Diabetic group, Group 3: Diabet + sham group, Group 4: Diabet + exenatide, Group 5: Diabet + empagliflozin, Group 6: Diabet + Quercetin, Group 7: Diabet + combination therapy group. AgNOR: Argyrophilic nucleolar organizer region. Relative AgNOR Area: Percentage ratio of the AgNOR–stained area to the total nuclear area. AgNOR data are expressed as mean ± SEM. Superscript letters ($^{a, b, c, d}$) indicate statistically significant differences between group means (P<0.05)

no statistically significant change was detected in the nucleus area (P>0,05). Considering the measurement results, the most pronounced improvement was observed in Group 7, followed by Groups 4, 5, and 6, respectively. Detailed measurement results are presented in TABLE II.

The DNA regions responsible for ribosomal RNA synthesis are referred to as nucleolar organizing regions (NORs). These regions can be visualized as black dots within the nucleus using silver staining methods and are defined as AgNORs. Quantitative assessment of the number and area of AgNORs is widely used as an indicator of cellular proliferation. Although the use of AgNOR analysis has declined with the development of immunohistochemical markers such as proliferating cell nuclear antigen (PCNA) and Ki67, the AgNOR method continues to offer significant advantages, including less susceptibility to fixation processes, low cost, and ease of application [48, 49].

Previous studies have shown that diabetes significantly reduces proliferative activity in the dentate gyrus region of the hippocampus [50, 51]. It is thought that the proliferation of granule cells in this region plays an important role in maintaining cognitive functions, particularly learning and memory processes, and that newly formed neurons are critical for the continuity of hippocampal functions [52, 53]. In this study, cellular proliferation was evaluated using the AgNOR staining method, and the findings showed that diabetes significantly reduced proliferative activity in the hippocampus, with this effect being more pronounced in the dentate gyrus region.

Glucagon–like peptide is an incretin hormone synthesized by L cells in the gastrointestinal tract. It plays an important role in regulating glucose homeostasis by stimulating insulin release and supporting the proliferation of pancreatic beta cells [54]. In addition to its metabolic effects, GLP–1 has been reported to exhibit neuroprotective properties and enhance learning and memory performance [55, 56].

However, the short biological half–life of natural GLP–1 has limited its clinical use and led to the development of more stable analogs. Exenatide, a GLP–1 receptor agonist, contributes to appetite regulation and blood glucose control in addition to increasing insulin release and suppressing glucagon secretion [16, 57]. Notably, exenatide has been shown to cross the blood–brain barrier and bind to GLP–1 receptors in the hippocampus [58].

In an experimental model of Alzheimer's disease, exenatide treatment was reported to increase the number of neurons in the CA1 and CA3 regions of the hippocampus [59]. Furthermore, Hamilton *et al.* [58] demonstrated that exenatide administration in rats with type 2 diabetes increased cellular proliferation in the dentate gyrus.

Similarly, Elsaeed *et al.* [35] reported that exenatide treatment increased cell numbers and reduced the apoptotic cell ratio in the hippocampus of diabetic animals. The findings obtained in this study are consistent with the literature and demonstrate that exenatide supports hippocampal cell proliferation and significantly reduces diabetes–related cell loss. Thus, it has been concluded that exenatide administration increases the cellular population in the hippocampus.

Empagliflozin is a SGLT–2 inhibitor used in the treatment of type 2 diabetes [15]. In recent years, studies have been conducted on the neuroprotective effects of empagliflozin. In a study reported that empagliflozin reduces oxidative stress caused by diabetes [60]. Another study reported that empagliflozin reduced histopathological damage in the hippocampus in an Alzheimer's disease model [61]. Similarly, another study reported that empagliflozin significantly reduced oxidative stress and antioxidant imbalance and improved the performance of animals in behavioral tests [62]. Empagliflozin not only provides glycemic control but also exhibits neuroprotective effects [63]. In this study, consistent with the literature data, empagliflozin was observed to exhibit neuroprotective activity and increase the number of healthy neurons.

Quercetin, a natural flavonoid, is a compound that has attracted attention as having therapeutic potential, particularly for its neuroprotective effects, due to its multifaceted pharmacological properties. Numerous studies have demonstrated that quercetin can protect neuronal cells by suppressing oxidative stress and reducing neuroinflammatory responses [19]. Furthermore, there is strong evidence that quercetin alleviates diabetes–related cognitive impairments and improves memory–related behaviors [64, 65, 66].

Kanter *et al*. [67] supported the neuroprotective capacity of this compound by demonstrating that quercetin administration significantly reduced neuronal degeneration. However, quercetin's limited ability to cross the blood–brain barrier is considered a factor that limits its effects on the central nervous system.

On the other hand, it is known that diabetes disrupts the integrity of the blood–brain barrier, increasing its permeability [68]. In this study, it was found that quercetin treatment reduced neuronal degeneration and increased the number of live neurons. This effect may be due to the increased blood–brain barrier permeability in diabetic conditions, allowing quercetin to reach the brain more easily and thus enhancing its neuroprotective effects.

Combining the efficacy of multiple drugs has been reported to provide more comprehensive and effective glycemic control without increasing the risk of hypoglycemia or other serious adverse effects. Studies have demonstrated that combination therapies not only improve glycemic control in the treatment of type 2 diabetes but also reduce blood pressure and may confer protective effects on multiple organs, including the liver and kidney in clinical studies, as well as the brain in preclinical models [35, 69, 70, 71]. Consistent with these findings, the present study also demonstrated that combination therapy produced superior outcomes.

CONCLUSION

In this study, histopathological changes caused by experimental diabetes in the hippocampal region were evaluated and the effects of exenatide, empagliflozin and quercetin treatments on these changes were investigated. In histopathological evaluation, shrunken, dark neurons with pyknotic nuclei were accepted as an indicator of neurodegeneration and were evaluated as apoptotic cells.

The evaluations revealed that diabetes causes significant neurodegeneration in the hippocampus, particularly by reducing the number of viable neurons. Exenatide and empagliflozin treatments were found to reduce this damage, while combined treatments were more effective than single treatments. Quercetin was also found to partially reduce cell loss, suggesting that this effect may be mediated by reducing oxidative stress.

This study provides preliminary evidence that hippocampal neuronal loss in experimental diabetes is assessed histopathologically and that some therapeutic agents may limit this damage. The fact that combination therapies, in particular, exhibit more favorable effects than single agents demonstrates significant potential for pharmacological research in this area. In this context, the anti–apoptotic and neuroinflammation–suppressing effects of Exenatide mediated via GLP–1 receptors the ability of Empagliflozin to reduce oxidative stress and mitochondrial dysfunction and the strong antioxidant and ferroptosis–modulating properties of Quercetin when considered together, suggest that the combined treatment may exert a multi–targeted and synergistic neuroprotective effect by simultaneously addressing key pathophysiological processes involved in diabetic neurodegeneration, including metabolic dysregulation, oxidative stress, and neuronal injury. Nevertheless, further molecular, immunohistochemical analyses and functional studies are required to fully elucidate the causal mechanisms underlying these effects.

Declaration of generative AI

During the preparation of this work the authors used AI in order to check grammar. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Data availability statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental data.

Credit authorship contribution statement

İlknur ÜNDAĞ: Conceptualization, Investigation, Methodology, Project administration, Resources, Writing – original draft, Visualization. Burak DİK: Conceptualization, Methodology, Project administration, Writing – Review & Editing. Hasan Hüseyin DÖNMEZ: Formal analysis, Investigation, Writing – Review & Editing, Supervision, Visualization. Yasemin KORKMAZ: Conceptualization, Methodology, Investigation

Conflict of Interest Statement

The authors state that they have no financial or personal relationships that could be viewed as potential conflicts of interest concerning this publication.

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