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Intracerebroventricular administration of the exercise hormone irisin or acute strenuous exercise alleviates epileptic seizure-induced neuroinflammation and improves memory dysfunction in rats

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Abstract

Background Status epilepticus is a common and potentially life-threatening neurological emergency with a high risk for cognitive and neurobiological impairment. Our aim was to evaluate the neuroprotective effects of centrally administered irisin and acute exhausting exercise against oxidative brain injury and memory dysfunction due to a pentylenetetrazole (PTZ)-induced single seizure. Male Sprague Dawley rats with intracerebroventricular (icv) cannulas were randomly divided into intraperitoneally (ip) saline-injected control and PTZ-injected (45 mg/kg) seizure groups. Both the control and PTZ groups were then treated with irisin (7.5 µg/kg, 2 µl, icv), saline (2 µl, icv) or were forced to an acute bout of strenuous exercise before the ip injection of saline (control) or PTZ. Seizures were evaluated using the Racine score. To evaluate memory performance, a passive avoidance test was performed before and after PTZ injection. Following euthanasia at the 24th hour of seizure induction, brain tissues were removed for histopathological examination and for evaluating oxidative damage, antioxidant capacity, and neurotransmitter levels.

Results Glutamate/GABA imbalance observed in PTZ rats was corrected by irisin administration ($p < 0.001/p < 0.01$), while irisin prevented the generation of reactive oxygen species and lipid peroxidation ($p < 0.05 - 0.001$) and replenished the antioxidant catalase and glutathione levels ($p < 0.01–0.01$) in the cerebral tissue, and reduced the histologically evident neuronal injury due to a single seizure ($p < 0.05 - 0.01$). Irisin also delayed the onset of seizures ($p < 0.05$) and improved memory dysfunction ($p < 0.05$), but did not affect the severity of seizures. The acute exhaustive swimming exercise completed before PTZ-seizure depressed glutamate level ($p < 0.001$), maintained the oxidant/antioxidant balance, alleviated neuronal injury ($p < 0.05 - 0.01$) and upregulated cerebral BDNF expression ($p < 0.05$).

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Conclusion In conclusion, acute high-intensity exercise or exogenously administered irisin provides neuroprotection by maintaining the balance of excitatory/inhibitory neurotransmitters and oxidant/antioxidant systems.

Keywords PTZ-seizure, Neuroinflammation, Oxidavite stress, Exercise, Irisin, Glutamate, GABA, BDNF

Background

Status epilepticus (SE) is a common and potentially life-threatening neurological emergency characterized by prolonged seizures that can lead to long-term neurobiological and cognitive consequences [1, 2]. Seizures are proposed to occur by an imbalance between the concentrations of the major inhibitory neurotransmitter gamma-aminobutyric acid (GABA) and the major excitatory neurotransmitter glutamate, leading to abnormal excessive or synchronous neuronal activity and cell death [3–5]. When glutamate receptors are overactivated by excitatory amino acids, calcium buffering is impaired, and subsequent calcium overload leads to the activation of enzymes that degrade nucleic acids, proteins, and cellular membranes, causing cell death and further excitotoxicity [6]. Experimental seizure models have shown that glial cells and neurons in seizure-related brain areas overexpress proinflammatory mediators, leading to mitochondrial oxidative stress and contributing to epileptogenesis via alterations in synaptic glutamate homeostasis [7]. In rodent models of epileptic seizures, reactive oxygen species (ROS), which accumulate during the acute phase of the seizure, play a crucial role in seizure-induced neurodegeneration and cognitive dysfunction [8]. Similarly, exercise was shown to protect against seizure-induced oxidative injury by inhibiting ROS generation in both the brain and skeletal muscles of rats [9]. Thus, targeting and suppressing oxidative stress could be valuable in the management of epileptic seizures and in the attenuation of epileptogenesis. Accordingly, various antioxidant and anti-inflammatory agents, as well as exercise, have been evaluated for their therapeutic potential in improving oxidative brain damage in rats with epileptic seizures [10–12].

Irisin, an exercise-induced myokine consisting of 112 amino acids, is produced by the cleavage of the precursor fibronectin type III domain-containing protein 5 (FNDC5) [13]. The transmembrane glycoprotein FNDC5 is widely expressed in skeletal muscle, adipose tissue, and various cerebral regions [14]. The major stimulants of irisin synthesis and release are short bouts of intensive exercise and cold-induced shivering [15–17]. Following acute strenuous exercise accompanied by the depletion of intracellular ATP in muscle tissue, FNDC5 expression and serum irisin levels are increased by the adenosine monophosphate-activated protein kinase (AMPK) or p38 mitogen-activated protein kinase (MAPK) peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) pathway [18–20]. The well-described physiological functions of irisin include its role in the browning of white adipose tissue, the regulation of insulin metabolism, and its anti-inflammatory and antioxidative effects [21]. Recombinant irisin injection or the endogenous release of irisin by exercise has been reported to reduce cerebral ischemia-induced neuronal injury and improve cognitive function in various neurodegenerative diseases [22–26]. In addition, irisin treatment was shown to exert significant neuroprotective effects in rats with kainic acid-induced SE via the upregulation of brain-derived neurotrophic factor (BDNF) expression [27]. Furthermore, the serum and brain tissue levels of FNDC5/irisin were shown to be elevated in pentylenetetrazole (PTZ)-induced epileptic rats [28], while children with idiopathic epilepsy exhibited increased serum irisin levels that correlated with the severity of their seizures [29], suggesting a possible compensatory release of irisin during epileptic seizures.

Based on studies showing that oxidative brain injury is a critical causative factor of epileptic seizure-induced neuronal damage [30] and reports verifying the neuroprotective potential of irisin in several neurodegenerative diseases by modulating neuroinflammation [23]. We hypothesized that over-contraction of the skeletal muscles during epileptic seizures, which causes release of irisin, could be a possible compensatory mechanism to protect against seizure-related neuronal injury. Our aim was to elucidate the putative neuroprotective effects of centrally administered irisin against oxidative brain injury and memory dysfunction due to PTZ-induced single seizures in rats. Secondly, we aimed to further evaluate whether acute exhausting exercise with exaggerated muscle contraction that mimics the over-contraction of the muscles as in epileptic seizure would have neuroprotective effects comparable to those of exogenous irisin administration.

Methods

Animals and study design

Female Sprague–Dawley rats (230–280 g, 12 weeks old; $n=48$) were obtained from the Acıbadem Mehmet Aydınlar University Animal Center. The animals were housed in a temperature (22±2 °C)-controlled and humidity (65–70%)-controlled room with constant light/dark (12/12 h) cycles and were fed standard rat pellets (Arden Research & Experiment, Turkey) and water *ad libitum*. Rats were housed with 4 rats in each cage during the acclimatization period of 1 week. After surgery, they were housed individually in solid-bottom cages

containing approximately 3 cm of soft corncob litter. Experiments were designed and performed in compliance with the Turkish law on the use of animals in experiments, and the guidelines of the New York Academy of Sciences were followed. All methods were performed in accordance with the relevant guidelines and regulations. The experiments performed in the present study also comply with the ARRIVE guidelines 2.0 (https://arriveguidelines.org/). All experimental protocols were approved by the Marmara University Animal Care and Use Committee (approval date and number; 16.11.2021, 86.2021.mar).

Stereotaxic surgery and experimental design

Under ketamine and xylazine (100 mg/kg and 12.5 mg/kg, respectively, intraperitoneally, ip) anesthesia, rats were administered intracerebroventricular (icv) cannulas via stereotaxic surgery. The scalps of the rats were shaved, and stainless steel cerebroventricular guide cannulas (22-gauge; Plastic Products, Roanoke, VA) were inserted into the right cerebral ventricles by using the coordinates (1.4 mm lateral, 0.8 mm posterior to bregma and 4.8 mm ventral to skull) given in the Paxinos and Watson rat brain atlas [31]. Cannulas were immobilized with 3 small screws, and dental acrylic filler was then used to cover the screws and skull. The rats were returned to their home cages and treated for three days with a subcutaneous injection of acetaminophen (Perfalgan; Bristol Myers Squibb; 0.1 mg/kg/day) for analgesia.

After a 1-week postoperative recovery period, all rats were randomly divided into two main groups: the saline-injected control group and the PTZ-injected seizure group (Fig. 1). Both the control and PTZ groups were then randomly divided into 3 subgroups ($n=8$ in each), which were pretreated with either recombinant irisin (7.5 µg/kg in 2 µl, icv; Phoenix Pharmaceuticals, Inc., Burlingame, USA) or saline (2 µl, icv) using a Hamilton syringe 5 min before the ip injection of saline or PTZ. The selected dose of irisin was previously shown to attenuate brain damage in a mouse model of stroke [24]. In the saline-pretreated control and PTZ groups, half of the animals were subjected to acute strenuous exercise before the icv injections were performed. To evaluate memory function, a passive avoidance test was performed before seizure induction and at 24th h following the epileptic seizure. Immediately after the second step of the passive avoidance test, euthanasia was conducted in accordance with the American Veterinary Medical Association (AVMA) guidelines and decapitation procedure was performed by using a guillotine with a sharp blade. Subsequently, brain (injured by epileptic seizure) and gastrocnemius muscle (injured by exaggerated muscle contraction by either seizure or exhaustive swimming) tissues were gently removed. Before decapitation, one rat in each group was randomly selected and injected with methylene blue via the icv route to confirm the correct cannula location.

Induction and scoring of seizures

Rats were injected intraperitoneally with a single dose of PTZ (45 mg/kg in saline; Sigma–Aldrich) to induce epileptic seizures [9]. PTZ is a noncompetitive selective

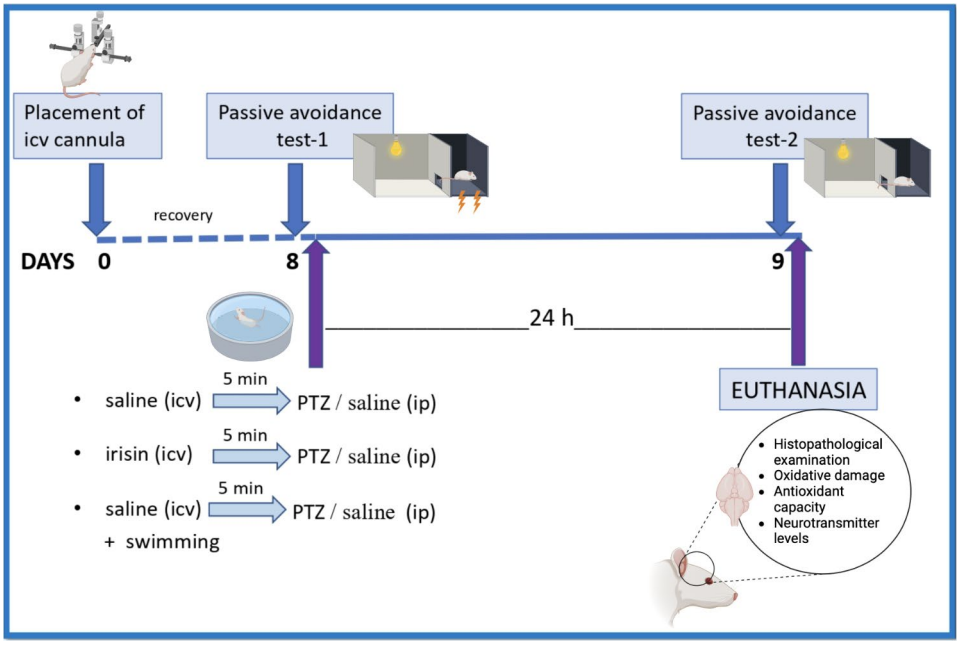

Fig. 1 Schematic representation of the applied procedures

blocker of the $GABA_A$ receptor commonly used to produce reproducible generalized seizures [32], and the rationale for choosing the intermediate convulsive dose was based on our previous observations [11, 12, 33]. PTZ-induced seizures were video-taped for the following 30 min in a Plexiglas observation box (38×30×25 cm). Afterwards, the recorded 30-min period was evaluated by using Racine's scoring (0–6), where *0: no seizure response, 1: immobility, eye closure, ear twitching, sniffing, facial clonus, 2: head nodding, severe facial clonus, 3: clonus of one forelimb, 3.5: clonus of both forelimbs without rearing, 4: bilateral forelimb clonus with rearing, 4.5: falling on a side without rearing, loss of righting reflex, generalized tonic–clonic seizures, 5: rearing and falling, 6: wild running, jumping, vocalization* [34].

Acute exhaustive exercise protocol

Since studies performed in human subjects suggest that acute strenuous exercise acutely and transiently increases serum irisin levels [35], a group of rats were subjected to acute exhaustive swimming exercise to induce the endogenous release of irisin. A modified version of the intense forced swimming exercise protocol was applied in rats with lead weights (8–9% of body weight) tied to their tails [36]. Swimming exercise was performed in a cylindrical plastic container (150×50×25 cm) filled with 50 cm deep water at 31–32 °C. The total duration of active swimming was 10 min, which was interrupted by 1-min resting periods, during which the rats were removed from the water when they showed signs of exhaustion. At the end of the swimming session, the rats were injected with icv saline, and 5 min later, PTZ or saline was administered intraperitoneally (Fig. 1).

Passive avoidance test

After a 7-day recovery period following cannula placement, the acquisition step of the passive avoidance test was performed, and it was repeated 24 h later to evaluate memory function (Fig. 1) [37, 38]. On the initial acquisition trial, rats were placed in the illuminated chamber of the passive avoidance apparatus (Northel, Istanbul; each chamber 20×20×20 cm) and were expected to move instinctively to the nonilluminated chamber [12]. After the rat passed into the nonilluminated area equipped with an electric grid floor, the guillotine door separating the illuminated and nonilluminated chambers was closed, and the rat was given an electrical foot shock (0.3–0.6 mA) for 5 s. Following the shock, the rat was returned to the illuminated chamber and then returned to its home cage. At the 24th h of the acquisition trial, the rat was placed into the illuminated chamber again to assess memory recall [38, 39]. The rats with intact memories avoided entering the nonilluminated chamber within 300 s (the cutoff point), while entering the nonilluminated chamber with a shorter latency indicated the presence of memory dysfunction.

High-performance liquid chromatography analysis of amino acids in cerebral tissue samples

HPLC analysis of glutamate and GABA was performed according to previously published methods [40]. Briefly, the brain tissue samples were homogenized in 0.1 M perchloric acid and centrifuged at 18,000 rpm for 20 min at 4 °C, after which the supernatants were analyzed. The analysis was performed by an Agilent 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany) consisting of a G1322A degasser, G1311A capillary pump, G1313A refrigerated autosampler, G1316A thermostatic column compartment, C18 column (ACE, Aberdeen, UK; C18, 250×4.6 mm × 5 µm) and G13215A fluorescent detector with the wavelengths set at 360 nm for excitation and 410 nm for emission. The mobile phase comprised 250 mM Na acetate (pH: 6.9), "HPLC-grade" deionized water, and methanol; 0.5% (v/v) tetrahydrofuran was added to all the solutions. The mobile phase was freshly prepared and degassed before each set. A gradient flow with an equilibration period of 10 min was applied at a flow rate of 0.5 ml/min. The equilibration period was performed with 47% sodium acetate buffer (250 mM, pH=6.9), 2% methanol, and 50% HPLC grade Millipore Milli-Q deionized water. The methanol ratio was increased to 100% in 28 min, remained at that rate for 2 minutes, and then returned to the equilibrium ratio. The temperature of the column was 25 °C. As external standards, L-glutamic acid and GABA dissolved in HCl were diluted with deionized water to obtain samples at three different concentrations (0.25 µM, 0.5 µM, 1 µM and 2.5 µM). The results were then adjusted to ng/mg tissue. Since the analysis was performed with a fluorescence detector, precolumn derivatization was performed to detect organic compounds. Precolumn derivatization was performed with o-phthaldialdehyde and 3-mercaptopropionic acid. Injections with an automated sampler were given in a 12 µl volume (10 µl sample or standard+4 µl derivatization solutions) at +4 °C. An autosampler was used to inject the samples. The retention times of glutamate and GABA were 8.20±0.10 min and 32.11±0.11 min, respectively. Linear regression yielded a good curve fit with an R^2=0.98.

Measurement of malondialdehyde and glutathione levels in cerebral tissue samples

The level of malondialdehyde (MDA), which is indicative of lipid peroxidation, and the level of the endogenous antioxidant glutathione (GSH) were measured in brain and muscle tissue samples. For the measurement of MDA levels, brain and muscle samples were homogenized with 10% trichloroacetic acid solution (TCA) with

an Ultra Turrax tissue homogenizer. After centrifugation (at 3000 rpm at 4 °C for 15 min), the MDA level was measured spectrophotometrically at 535 nm. The results are expressed in nmol/g tissue [41]. The same homogenates were used for the measurement of GSH using the modified Ellman method. The amount of GSH was measured spectrophotometrically at 412 nm and is expressed as µmol/g tissue.

Measurement of superoxide dismutase and catalase activity levels in cerebral tissue samples

Superoxide dismutase (SOD) activity in the brain and muscle samples was measured using the ability of the SOD enzyme to enhance the photooxidation of o-dianisidine via the sensitization of riboflavin. The absorbances were measured spectrophotometrically at 460 nm, and the results were calculated using a standard curve prepared with bovine SOD (Sigma Chemical Co., catalog no. S-2515, 3000 units). The measurement of catalase (CAT) activity was based on the hydrolysis of H_2O_2 to H_2O at 240 nm in the first minute. The enzymatic activity of both SOD and CAT was expressed as units/mg tissue [42].

Measurement of reactive oxygen species in cerebral tissue samples by chemiluminescence assay

The chemiluminescence (CL) assay is a noninvasive and commonly utilized method to directly assess the levels of ROS and nitric oxide (NO). A probe for lucigenin (bis-N-methylacridinium nitrate; Sigma, St. Louis, MO) specifically detects O_2•−radicals, while a luminol probe (5-amino-2,3-dihydro-1,4-phthalazinedione) is frequently used for the detection of hydroxyl, hydrogen peroxide and hypochlorite radicals. CL measurements of ROS in the brain samples were performed by adding luminol or lucigenin probes at 0.2 mM final concentration each, while NO was measured by adding K_2CO_3 (0.4 mM), desferrioxamine (60 mM), H_2O_2 (4 mM) and luminol sodium salt (3.6 mM) to the tubes containing brain tissue samples [43]. Luminol, lucigenin and NO levels were recorded at room temperature by a luminometer (Junior Lumat LB 9509; EG&G Berthold, Germany), and CL levels were calculated by linear approximation and expressed as the area under the curve (AUC) of relative light units per milligram of tissue [44].

Histological examination of cerebral tissue samples

The brain and skeletal muscle tissue samples were dissected from animals in each group and fixed in 4% paraformaldehyde. After dehydration (with 70%, 80%, 90%, and 100% alcohol), the tissues were passed through xylene and embedded in paraffin. Four-to-five micrometer sections were cut from the paraffin blocks using a rotary microtome and deparaffinized in xylene, and hematoxylin-eosin staining was performed for morphological assessment. Histopathological evaluation was performed with an Olympus BX51 microscope (Olympus Inc., Tokyo, Japan) by a histologist who was blinded to the study groups. The severity of neuronal damage in the cortex and hippocampal dentate gyrus (DG) and cornu ammonis 3 (CA) regions was scored semi-quantitatively as follows: *0=no damage, 1=mild damage, 2=moderate damage, and 3=severe damage.*

For immunohistochemical examination, the tissue sections were deparaffinized, hydrated, treated with 0.3% hydrogen peroxide to inhibit endogenous peroxidase activity, and then exposed to antigen repair with citrate at high pressure. Later, the brains were incubated with an anti-BDNF (Abcam ab213323, rabbit monoclonal, 1:10000) antibody. The slides were incubated with 3,3'-diaminobenzidine tetrahydrochloride dihydrate (Invitrogen 002020; DAB Plus Substrate Kit) for 5 min after the addition of biotinylated secondary antibody and horseradish peroxidase (HRP) streptavidin (Invitrogen 859043, Histostain Plus Broad Spectrum). Then, the sections were counterstained with Mayer's hematoxylin and mounted with entellan (Merck, Darmstadt, Germany). Sections were semi-quantitatively scored in terms of staining intensity from 0 to 3, where *0: none; 1: mild; 2: moderate; and 3: severe.*

Statistical analysis

Using G*Power 3.1.9.4 and the following parameters (ANOVA, effect size d=0.05, alpha=0.05), it was determined that the sample size needed to obtain a power of at least 0.95 was 8 animals for each group. Statistical analysis was performed using GraphPad Prism 10.0 (GraphPad Software, San Diego, CA, USA). All the data are expressed as the means±SEMs. Datasets that did not have a normal distribution, nonparametric statistics (Kruskal-Wallis, followed by Dunn's multiple comparisons test) was applied. Otherwise, for the experiments that had multiple variables, a two-way ANOVA was performed. The significance level of statistical tests was set to 0.05.

Results

In all the seizure-induced rats that received either saline or irisin or had exercised prior to PTZ administration, seizure scores were similar (3.94±0.50, 3.44±0.44, 3.71±0.24, respectively; $p<0.05$; Fig. 2A). However, compared to those in the saline-treated PTZ group, tonic–clonic seizures occurred significantly later in both the irisin-treated and pre-exercised rats ($p<0.05$; Fig. 2B). At the initial acquisition step of the passive avoidance test, all rats entered the dark chamber shortly after they were placed in the illuminated compartment, and the latency periods were not significantly different among the experimental groups (data not shown); these groups were then

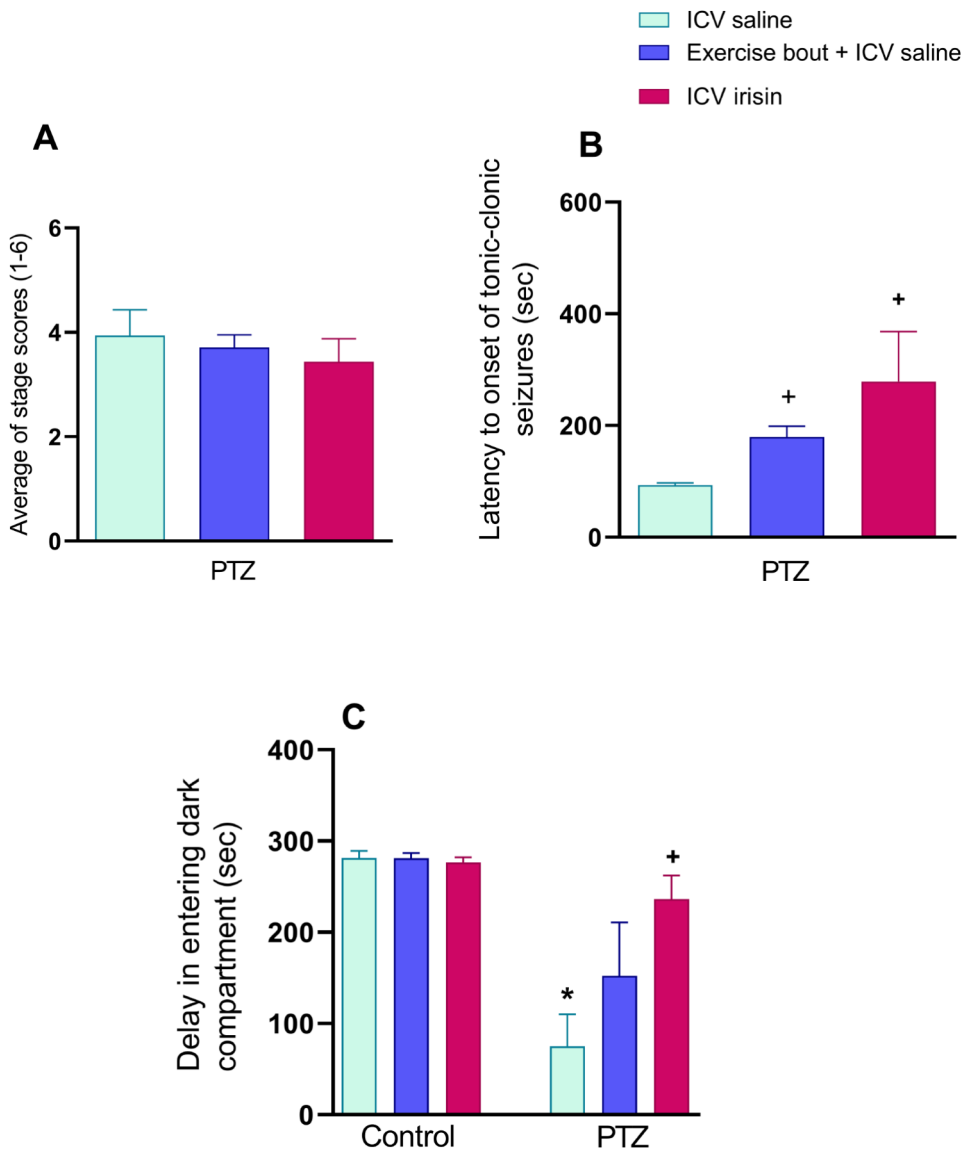

Fig. 2 (**A**) Average Racine stage scores. (**B**) Latency to the onset of a generalized tonic–clonic seizure following PTZ injection. (**C**) Delay in the entrance to the dark compartment in the passive avoidance test. $+p < 0.05$, compared to the PTZ group treated with icv saline; $*p < 0.05$, compared to the icv saline-treated control group. $n = 8$ in each group

randomly assigned to different treatment strategies. At the recall step of the passive avoidance test performed at 24 h after the PTZ injection, the control rats treated with either of the regimens avoided entering the dark chamber where they received electrical shock, while the saline-treated PTZ group entered the dark chamber in a significantly shorter period of time ($p < 0.05$; Fig. 2C), indicating memory dysfunction. However, the time of entry to the dark chamber was significantly delayed in the irisin-treated PTZ group ($p < 0.05$) and was not different from that in the control group, indicating a noteworthy improvement in memory function. On the other hand, pre-exercised rats with PTZ-induced seizures entered the dark compartment for a longer period of time, which was not significantly different from what was observed in the control or saline-treated PTZ groups, indicating a partial improvement in seizure-impaired memory.

Compared with the measured neurotransmitter levels in the brain tissues of the saline-treated control group, the levels of the inhibitory neurotransmitter GABA were significantly decreased in the saline-treated PTZ-treated rats ($p < 0.01$; Fig. 3A), while the excitatory glutamate levels were increased by 15-fold ($p < 0.001$; Fig. 3B), indicating that an imbalance between excitatory and inhibitory neurotransmitters predisposes them to the development of glutamate excitotoxicity. Acute bouts of exhaustive exercise prior to seizure induction had no significant effect on seizure-related GABA levels, but glutamate

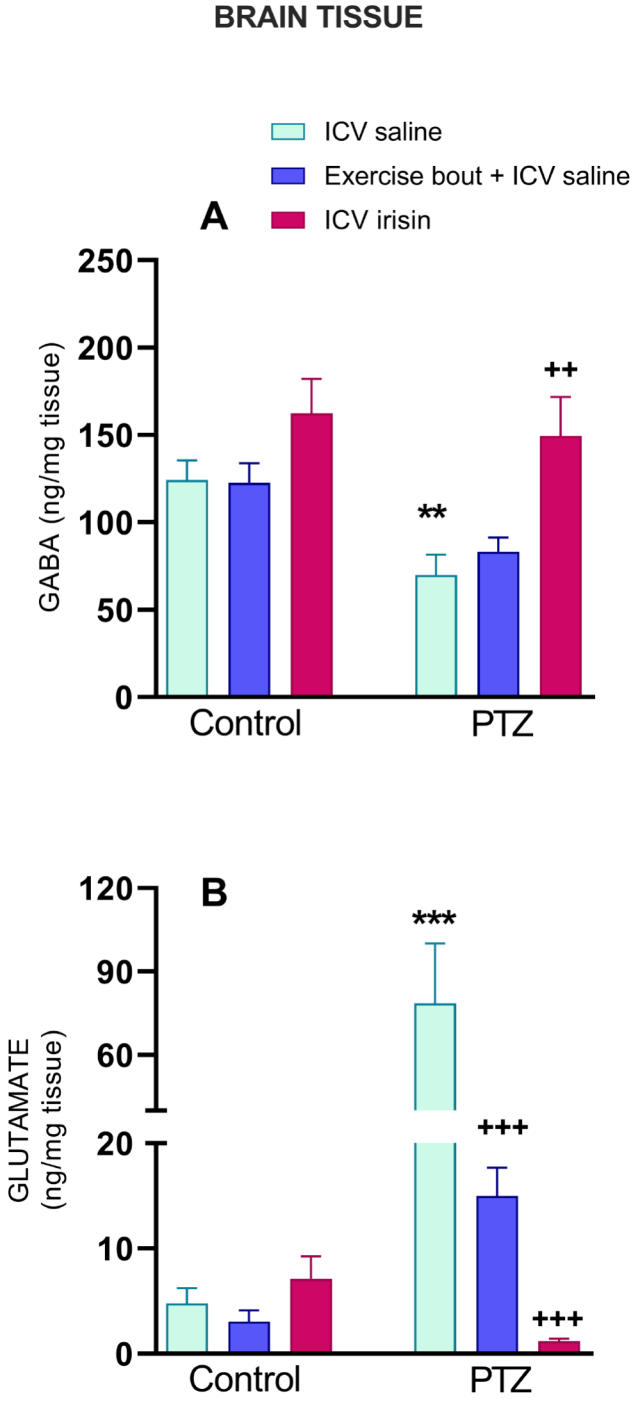

Fig. 3 (**A**) GABA and (**B**) glutamate levels in brain tissue. ++$p<0.01$, +++$p<0.001$, compared to the PTZ group treated with icv saline; **$p<0.01$, ***$p<0.001$, compared to the icv saline-treated control group. $n=8$ in each group

levels were significantly reduced when epileptic seizures were preceded by strenuous exercise ($p<0.001$), which suggests that exhaustive exercise may have an inhibitory impact on seizure-induced glutamate excitotoxicity. On the other hand, icv administration of irisin before the induction of an epileptic seizure decreased the concentration of glutamate ($p<0.001$) and increased the GABA level ($p<0.01$), restoring GABA and glutamate homeostasis.

The level of MDA, which is the end-product of lipid peroxidation, was significantly greater in the brain tissues of saline-treated PTZ-treated rats than in those of saline-treated control rats ($p<0.05$), whereas cerebral MDA levels in pre-exercised and irisin-treated rats were not different from those in the respective control groups (Fig. 4A). In accordance with the elevated lipid peroxidation, chemiluminescence assays revealed that the generation of NO, superoxide (detected by lucigenin), hydroxyl, hydrogen peroxide and hypochlorite radicals (detected by luminol) was elevated in the brain tissues of seizure-induced rats that were treated with saline ($p<0.01-0.001$; Fig. 4B, 4 C, 4D). On the other hand, irisin abolished the production of ROS and NO in cerebral tissue ($p<0.01-0.001$), indicating the radical scavenging activity of irisin in the seizure-injured brain. Similarly, acute bouts of high-intensity exercise before seizures also decreased ROS levels ($p<0.01-0.001$), but the reduction in NO did not reach statistical significance. In parallel with the observed oxidative stress in the brain tissue, the activities of the antioxidants CAT ($p<0.05$) and GSH ($p<0.01$) were significantly reduced, while the depletion of CAT and GSH was prevented by irisin treatment ($p<0.01$; Fig. 4E F). In rats that swam acutely before PTZ-induced seizures, the cerebral levels of CAT and GSH were not different from those in control rats, but PTZ-induced seizures preceded by swimming decreased cerebral SOD activity ($p<0.05$), which was not altered by seizures per se (Fig. 4G). The levels of MDA, CAT, GSH and SOD in the gastrocnemius muscles of control and PTZ-induced rats were similar, verifying that neither epileptic seizures nor acute swimming exercise resulted in oxidative injury to skeletal muscle due to exaggerated contractile activity (Table 1).

Hematoxylin and eosin staining of the cortices and hippocampal DG and CA3 regions of the control groups revealed the regular morphology of the neurons, and no difference was detected between the control groups that underwent short bouts of swimming exercise and the control groups that were treated with either saline or irisin before epileptic seizures were induced (Figs. 5 and 6). Marked neuronal degeneration was observed in the cortices and both hippocampal regions of the rats in the saline-treated PTZ group, as indicated by high histological damage scores ($p<0.01-0.001$). Short-term intensive exercise or irisin treatment prior to PTZ reduced neuronal damage in the cortices and hippocampal DG and CA3 regions of seizure-induced rats ($p<0.05-0.01$).

BDNF-positive staining was detected in all brain regions, while the BDNF immunoreactivity scores in the

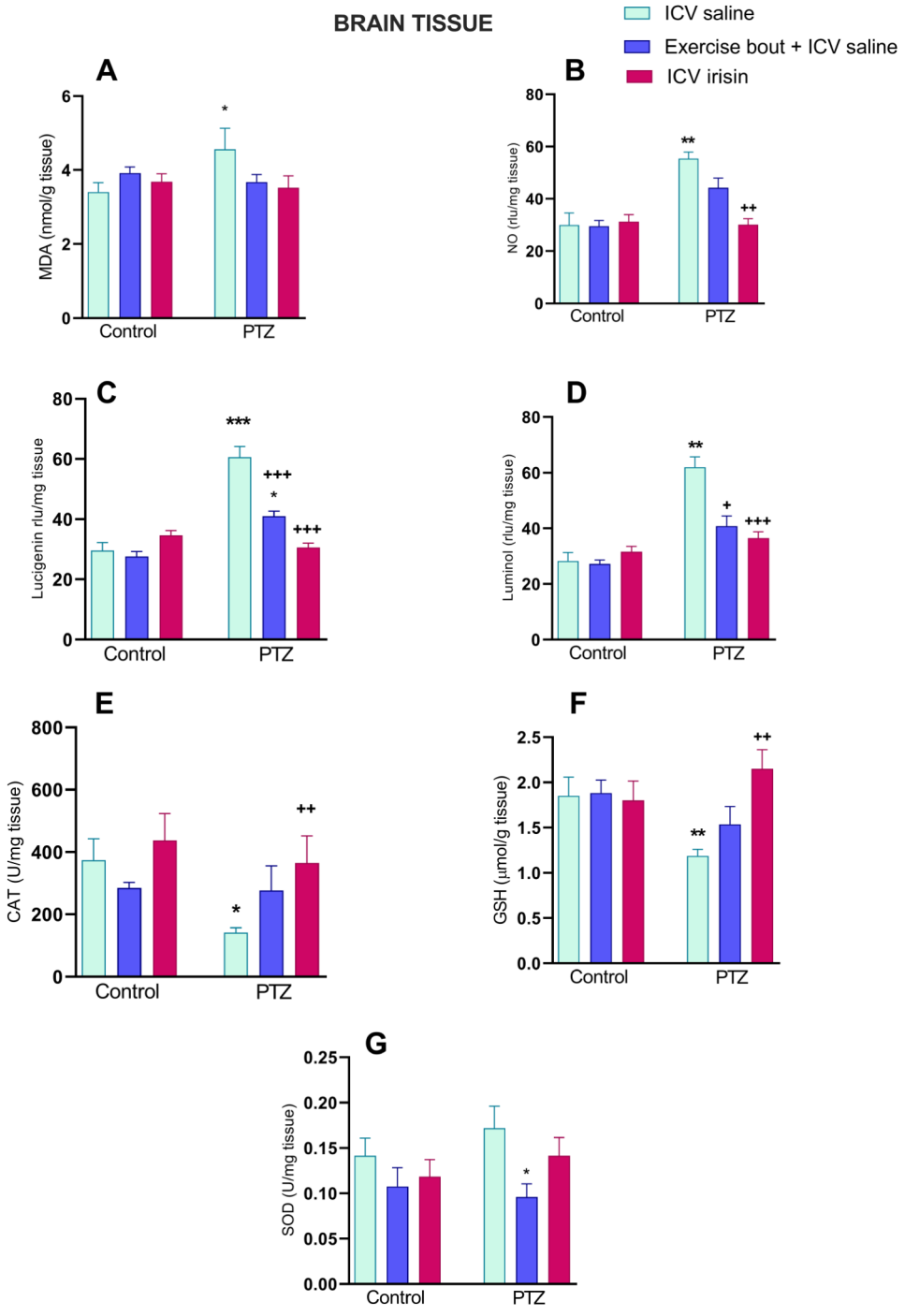

Fig. 4 (**A**) Malondialdehyde, (**B**) nitric oxide, (**C**) lucigenin, (**D**) luminol, (**E**) catalase, (**F**) glutathione and (**G**) superoxide dismutase levels in brain tissue. $+p<0.05$, $++p<0.01$, $+++p<0.001$, compared to the PTZ group treated with icv saline; $*p<0.05$, $**p<0.01$, $***p<0.001$, compared to the icv saline-treated control group. $n=8$ in each group

cortex and hippocampus were lower in the saline-treated PTZ group, without reaching a statistical significance (Figs. 5 and 7). Although an acute bout of intensive exercise per se did not alter BDNF immunoreactivity in the control rats, BDNF expression in the cortex and hippocampus was significantly increased before PTZ induction ($p<0.05$), and the decrease in BDNF expression due to PTZ seizures was reversed to control levels. On the other hand, irisin administration resulted in increased hippocampal BDNF expression compared to that in the saline-treated PTZ group, but these increases were not statistically significant.

Table 1 The levels of malondialdehyde (MDA), antioxidants catalase (CAT), glutathione (GSH), and superoxide dismutase (SOD) in the gastrocnemius muscles of control and PTZ-induced rats treated with either irisin, saline or that have completed an acute exhaustive exercise

	Control			PTZ		
	Saline	Exercise	Irisin	Saline	Exercise	Irisin
MDA (nmol/g tissue)	28.9 ± 1.7	32.7 ± 3.1	30.9 ± 3.2	31.5 ± 4.2	33.7 ± 2.3	33.1 ± 1.4
CAT (units/mg tissue)	177.7 ± 15.9	178.8 ± 37.1	114.6 ± 31.6	101.3 ± 24.4	131.8 ± 11.3	142 ± 23.4
GSH (µmol/g tissue)	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
SOD (units/mg tissue)	34.6 ± 6.4	39 ± 4.6	26.3 ± 4.7	27.9 ± 3.3	32.1 ± 3.3	24.4 ± 0.8

Discussion

The findings of the present study revealed that irisin administration immediately before PTZ-induced seizures corrected the glutamate/GABA imbalance, prevented the generation of ROS and lipid peroxidation, replenished the levels of the antioxidants CAT and GSH in brain tissue and reduced histologically evident neuronal injury due to a single SE seizure. Moreover, irisin delayed the onset of SE following PTZ injection and improved seizure-associated memory dysfunction but had no effect on the severity of seizures. Similar to exogenously administered irisin, a single acute exhaustive swimming exercise completed before PTZ-induced seizure also maintained the oxidant/antioxidant balance, alleviated neuronal injury, partially improved memory and upregulated BDNF expression. Thus, centrally administered irisin or high-intensity exercise, which is known to release irisin, exerts neuroprotective effects by maintaining the balance of excitatory/inhibitory neurotransmitters and oxidant/antioxidant systems. These findings provide a novel understanding of the complex neuroprotective mechanisms associated with irisin administration and acute strenuous exercise in the setting of epileptic seizures.

It is widely recognized that physical activity has beneficial effects on human well-being and prevents the occurrence of several chronic diseases and premature death [45]. Although the exact underlying mechanisms have not been fully elucidated, neurotrophins and myokines are described as contributing factors to the positive effects of physical activity [46, 47]. Irisin, which is a myokine secreted after intense exercise, has been shown to contribute to the neuroprotective effects of physical exercise via its mediatory effect on the "skeletal muscle-brain axis" [13, 22]. Both human trials and animal experiments have revealed that serum levels of irisin increase immediately after an acute bout of exhausting exercise [35, 48, 49]. On the other hand, serum irisin levels are significantly elevated in children with uncontrolled seizures [29], resulting in generalized maximal muscle contractions. Since exercise and irisin have been postulated to have neuroprotective effects [50], it is possible that both exercise and irisin have the potential to alleviate epileptic seizure severity and seizure-induced neuronal degeneration. Accordingly, in the present study, histologically evident neuronal degeneration in the cortical and hippocampal regions of saline-treated PTZ-treated rats was significantly reduced when the rats were pretreated with irisin or forced to exercise before seizures. These findings are consistent with recent research highlighting the neuroprotective effects of irisin and exercise on preserving and protecting neurons in neurodegenerative diseases [25, 47, 51–53], including chronic epilepsy. However, these earlier studies did not evaluate the ameliorative effect of irisin or exercise on single seizure-induced oxidative injury and memory impairment.

It was reported that experimental Alzheimer's disease was associated with reduced FNDC5/irisin levels in the hippocampi and cerebrospinal fluid of mice, while treatment with recombinant irisin was effective in preserving synaptic plasticity and preventing the formation of memory defects [26]. Moreover, the neuroprotective effect of physical exercise on synaptic plasticity and memory in these mice with Alzheimer's disease was abolished by using an anti-FNDC5 antibody, suggesting that irisin acts as a mediator of the beneficial effects of exercise. Irisin also supports the development of learning function by activating hippocampal neurogenesis, preventing neuronal damage caused by oxidative stress and improving synaptic plasticity [54], while its exogenous administration was shown to reduce the occurrence of spontaneous seizures, neurodegeneration, and memory defects in a rat model of kainic acid-induced chronic spontaneous epilepsy [51]. In support of these animal studies, research in epileptic patients also indicates that exercise can improve learning and memory functions [55]. Accordingly, our findings also revealed that irisin-treated PTZ-treated rats exhibited a significant improvement in memory function, while preexercised rats demonstrated a partial amelioration of seizure-induced memory deficits. Since memory impairment presents a significant challenge for individuals with epilepsy, improving memory via either exogenous irisin or its endogenous release by exercise offers

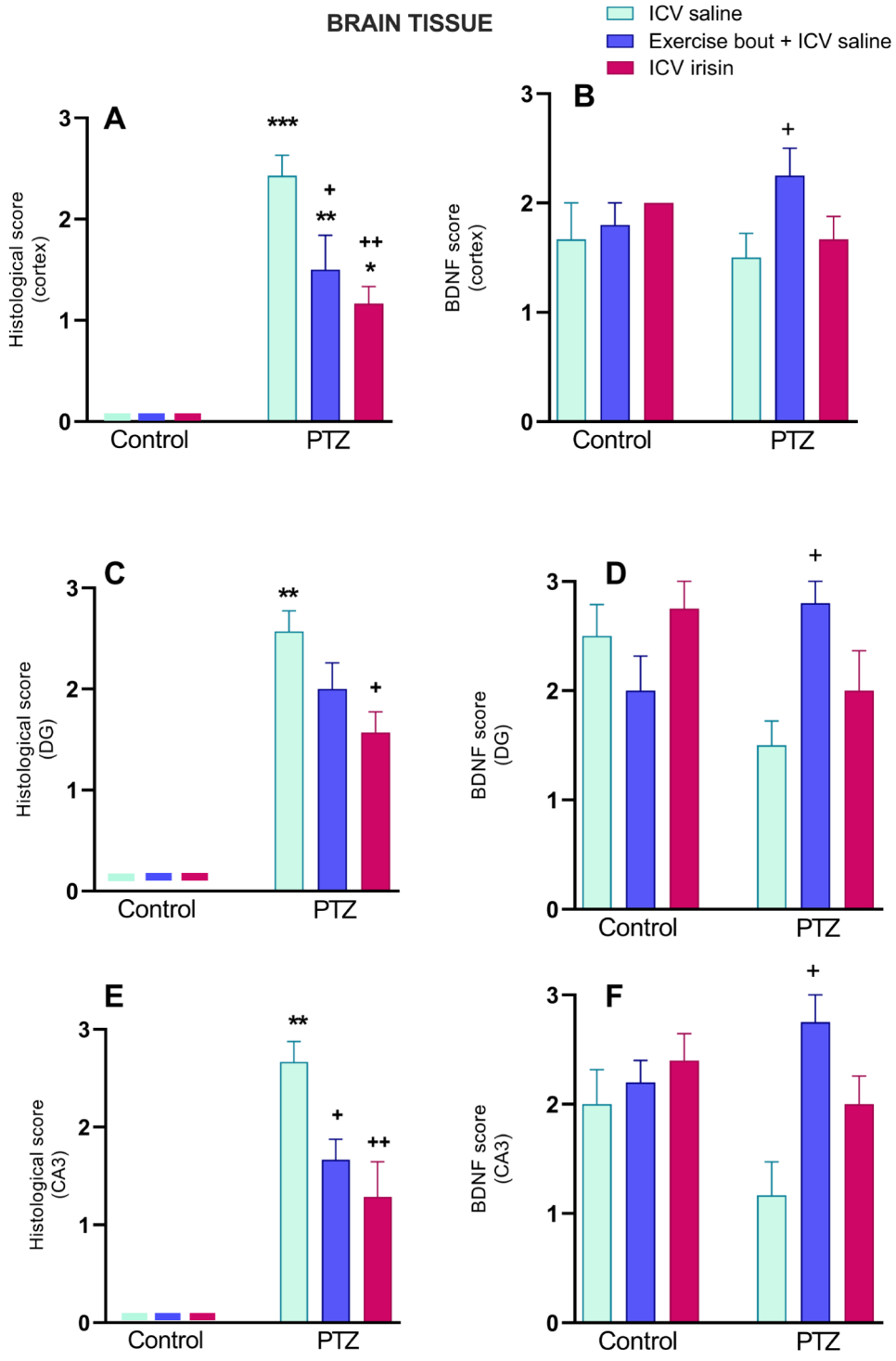

Fig. 5 Histopathological damage scores and immunohistochemical staining scores of BDNF in brain tissues. **A**-**B**) Cortex, **B**-**D**) dentate gyrus, **E**-**F**) cornu ammonis 3. $+p < 0.05$, $++p < 0.01$, compared to the PTZ group treated with icv saline; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared to the icv saline-treated control group. $n = 8$ in each group

a complementary treatment option for seizure-induced memory dysfunction.

An imbalance in excitatory/inhibitory neurotransmitters with elevated glutamate and reduced GABA concentrations in the brain has been implicated in ictogenesis and epileptogenesis [4]. On the other hand, it was shown that a single bout of vigorous exercise in healthy human volunteers was effective in increasing cortical

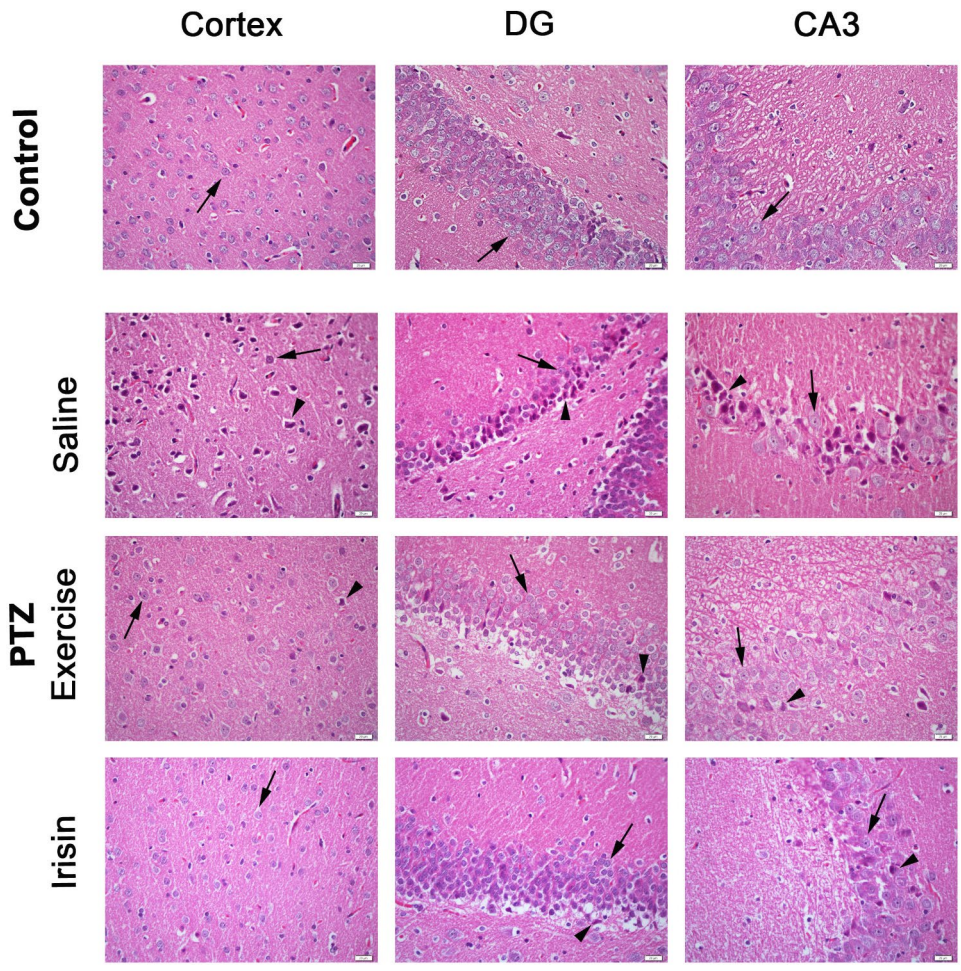

Fig. 6 Representative micrographs of the cortex, hippocampal dentate gyrus and CA3 region in the experimental groups. The black arrows indicate normal neuronal morphology, and the black arrowheads indicate damaged neurons. Micrographs of the saline-treated control group represent all the control groups treated with either saline or irisin or subjected to exercise, all of which demonstrated normal neuronal morphology. Hematoxylin and eosin staining. Scale bars = 20 µm

glutamate and GABA levels [56], suggesting a modulatory effect of exercise and exercise-induced irisin release in correcting the neurotransmitter imbalances that occur in neurodegenerative disorders [57]. In our study, a single epileptic seizure induced by PTZ, a noncompetitive antagonist of the GABA-A receptor, was accompanied by decreased cerebral levels of GABA and an exaggerated increase in excitatory glutamate levels, which was also evident in pentylenetetrazole-kindled mice [58]. In the present study, when rats performed strenuous exercise before PTZ injection, glutamate levels were significantly reduced, suggesting a potential role for exercise in alleviating glutamate excitotoxicity. Central administration of irisin also reduced glutamate levels with a concomitant increase in GABA levels, restoring the neurotransmitter balance to the control level. In accordance with our results, chronic moderate treadmill exercise was shown to reduce the severity of seizures with latency, which was attributed to increased expression of key neurotransmitter-related genes such as GAD65 and GABA-A receptor α1 in the hippocampus [52]. Although neither an exercise session nor central irisin administration changed the severity of seizures, both single applications were effective in delaying the occurrence of seizures and reversing seizure-induced GABA/glutamate imbalance, suggesting that repetitive exercise plays a critical role in preventing epileptogenesis by modulating neurotransmitter homeostasis.

Evidence from seizure-induced rodent models has demonstrated that oxidative stress during seizures promotes hyperexcitability, excitotoxicity and epileptogenesis [59]. The generation of ROS and reactive nitrogen species (RNS) in the early phase of a seizure results in reactive gliosis, mitochondrial DNA damage, neurodegeneration, and hyperexcitability with a reduced seizure threshold [60]. Although acute exercise is known to induce oxidative stress [61], exercise also stimulates the upregulation of antioxidant defenses and protects

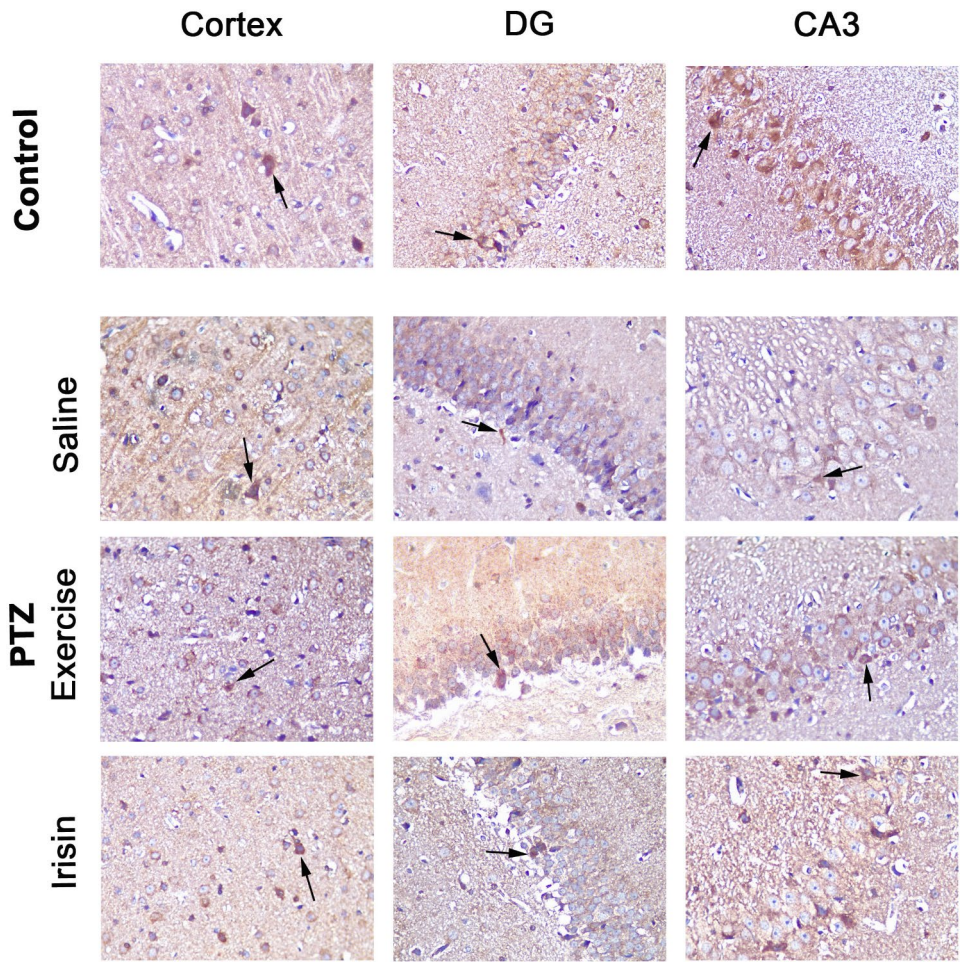

Fig. 7 Brain-derived neurotrophic factor (BDNF) immunoreactivities in the cortex, hippocampus DG and CA3 regions of the brain tissues of the experimental groups. Micrographs of the saline-treated control group represent all the control groups treated with either saline or irisin or subjected to exercise, all of which demonstrated identical BDNF immunoreactivity. The black arrows indicate BDNF immunoreactivity. Scale bars: 40 µm

against exposure to subsequent prooxidant settings [62]. Although proinflammatory cytokines promote the release of irisin from muscle tissue [63], irisin can exert anti-inflammatory effects to protect numerous tissues against stress conditions by reducing the release of TNF-a, interleukin (IL)-6 and NF-kB [46, 64, 65]. Moreover, irisin can stimulate the synthesis of antioxidant enzymes, including glutathione peroxidase (GPX-1), CAT and SOD [66]. Our current data showed that acute strenuous swimming exercise in control rats did not alter the generation of ROS or NO and had no significant effect on the brain levels of CAT, GSH or SOD. However, this acute bout of strenuous swimming exercise or central administration of irisin abolished the PTZ-induced production of ROS and NO, while depletion of CAT and GSH levels due to seizures was prevented. Consistent with our results in rats with PTZ-induced epileptic seizures, previous studies in several other tissues have reported that exogenous irisin treatment significantly reduced ROS levels [67–69]. On the other hand, we have previously demonstrated that treadmill exercise for 10 days before PTZ-induced seizures offered preconditioning protection against ROS generation and oxidative damage in both the brain and skeletal muscles of rats [9]. However, in the current study, we observed that PTZ-induced generalized muscle contractions preceded by exercise or central administration of saline or irisin had no effect on the oxidative state of the gastrocnemius muscle, suggesting that neither exercise nor seizure contractions had a noxious effect on skeletal muscle. Taken together, these findings suggest that the combination of antiseizure drugs with antioxidants or the addition of the nonpharmacological therapeutic effect of exercise to conventional antiepileptic agents might increase the effectiveness of antiseizure treatment and/or limit epileptogenesis.

The neurotrophin BDNF, which participates in the maintenance of neuronal proliferation, plasticity and survival, was postulated to be one of the underlying mechanisms involved in the beneficial effects of exercise and irisin on neuroinflammation and memory dysfunction

[26, 46, 70]. Wrann et al. [71] reported that voluntarily performing 30 days of endurance exercise on a running wheel elevates PGC-1α, FNDC5 and BDNF expression in the mouse hippocampus, which is the brain area associated with learning and memory functions. Our data showed that PTZ reduced hippocampal BDNF immunoreactivity without reaching statistical significance, but a single 10-min session of vigorous swimming prevented BDNF downregulation, suggesting that repetitive exercise sessions are necessary for a more evident stimulatory effect of exercise on BDNF expression and memory function. On the other hand, central irisin administration (7.5 µg/kg) before PTZ-induced seizure, which effectively improved memory, had a statistically nonsignificant impact on BDNF expression in the brain. Since it was previously shown that a single irisin treatment (49.5 µg/kg) applied before the induction of SE with kainic acid results in increased BDNF expression in the cortex and hippocampus of rats [27], it appears that the dose of irisin and the characteristics of the SE model could be critical for the cerebral BDNF response to exogenous irisin. Nevertheless, the current study is unique in that it compared irisin and exercise and demonstrated that both treatments had similar neuroprotective effects on single seizure-induced cerebral oxidative damage. Although our study provides original insight into the similarities between exercise and irisin in their neuroprotective effects, a major limitation of our study is the lack of circulating irisin measurements, which would have provided the opportunity to compare the dose of exogenously administered irisin with the amount of irisin released by a 10-min exhaustive swimming. A single effective dose of irisin, which was previously shown to be neuroprotective, was used in the current study; but several other doses may be assessed to enlighten the dose-dependent effects of irisin.

In conclusion, the release of irisin or irisin by vigorous exercise provides neuroprotection by maintaining the balance of oxidative/antioxidative systems along with excitatory/inhibitory neurotransmitters. Although further translational studies are necessary, the present findings are encouraging for the putative therapeutic potential of irisin and exercise regimens in individuals with epilepsy.

Abbreviations

DG	Hippocampal dentate gyrus
CA	Cornu ammonis 3
PTZ	Pentylenetetrazole
ICV	Intracerebroventricular administration
IP	Intraperitoneal administration
GABA	Gamma-Aminobutyric Acid
BDNF	Brain-derived neurotrophic factor
SE	Status epilepticus
ROS	Reactive oxygen species
FNDC5	Fibronectin type III domain-containing protein 5
AMPK	Adenosine monophosphate-activated protein kinase
MAPK	Mitogen-activated protein kinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
HPLC	High-Performance Liquid Chromatography Analysis
MDA	Malondialdehyde
GSH	Glutathione
SOD	Superoxide dismutase
CAT	Catalase
CL	Chemiluminescence
NO	Nitric oxide
RNS	Reactive nitrogen species
AUC	Area under the curve
HRP	Horseradish peroxidase
TNF-a	Tumor Necrosis Factor Alpha
GPX	Glutathione peroxidase
AVMA	American Veterinary Medical Association

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Author contributions
ZNOK and BY conceived and designed the experiments and took responsibility for the integrity of the work as a whole; ZNOK, TA, CH, EU, MKK, MFY, TO, ZU, DA, MY, and ZG conducted the experiments; ZNOK and TA contributed materials and analysis tools; ZNOK, TA and BY drafted the main manuscript; all authors reviewed and approved the final manuscript.

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Data availability
The authors declare that the data supporting the findings of this study are available within the paper. Should raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Declarations

Ethical approval and consent to participate
The experiments were performed in compliance with the Turkish law on the use of animals in experiments, and the principles and guidelines developed by the New York Academy of Sciences were followed. All experimental procedures were approved by the MU Animal Care and Use Committee (approval code: 86.2021.mar).

Consent for publication
BY gives her consent for the publication of identifiable details, which can include microphotograph(s) and/or graphs and/or and/or details within the text ("Material") to be published in the BMC Neuroscience.

Competing interests
The authors declare no competing interests.

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