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Original article

Therapeutic potential of endothelial progenitor cells in a rat model of epilepsy: Role of autophagy

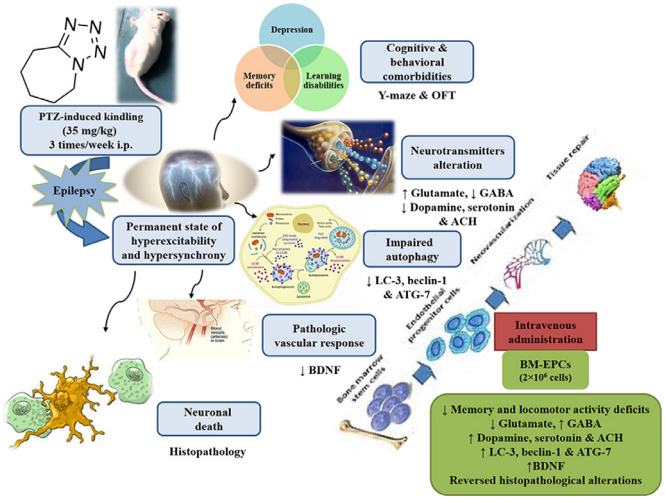
Shimaa O. Ali [a,*], Nancy N. Shahin [a], Marwa M. Safar [b,c], Sherine M. Rizk [a]

[a] Biochemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, Cairo, Egypt
[b] Pharmacology and Toxicology Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, Cairo, Egypt
[c] Pharmacology and Biochemistry Department, Faculty of Pharmacy, The British University in Egypt, El-Sherouk City, Cairo, Egypt

HIGHLIGHTS

- This is the first report showing EPCs therapeutic effects in PTZ-induced epilepsy.
- Intravenously administered EPCs homed into the epileptic rat hippocampus.
- EPCs amend the memory and locomotor activity deficits related to epilepsy.
- EPCs ameliorate epilepsy-associated alterations in neurotransmitters and autophagy.
- EPCs mitigate concomitant histological and vascular anomalies.

GRAPHICAL ABSTRACT



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ABSTRACT

Epilepsy is one of the most well-known neurological conditions worldwide. One-third of adult epileptic patients do not respond to antiepileptic drugs or surgical treatment and therefore suffer from the resistant type of epilepsy. Stem cells have been given substantial consideration in the field of epilepsy therapeutics. The implication of pathologic vascular response in sustained seizures and the eminent role of endothelial progenitor cells (EPCs) in maintaining vascular integrity tempted us to investigate the potential therapeutic effects of EPCs in a pentylenetetrazole (PTZ)-induced rat model of epilepsy. Modulation of autophagy, a process that enables neurons to maintain an equilibrium of synthesis, degradation and subsequent reprocessing of cellular components, has been targeted. Intravenously administered EPCs homed into the hippocampus and amended the deficits in memory and locomotor activity. The cells mitigated neurological damage and the associated histopathological alterations and boosted the expression of brain-derived neurotrophic factor. EPCs corrected the perturbations in neurotransmitter activity and enhanced the expression of the downregulated autophagy proteins light chain protein-3 (LC-3), beclin-1, and autophagy-related gene-7 (ATG-7). Generally, these effects were comparable to those achieved by the reference antiepileptic drug, valproic acid. In conclusion, EPCs may confer therapeutic effects

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* Corresponding author.
E-mail address: shimaa.omar@pharma.cu.edu.eg (S.O. Ali).

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against epilepsy and its associated behavioural and biochemical abnormalities at least in part via the upregulation of autophagy. The study warrants further research in experimental and clinical settings to verify the prospect of using EPCs as a valid therapeutic strategy in patients with epilepsy.

Introduction

With 50 million people affected worldwide, the World Health Organization (WHO) ranks epilepsy among the best-known and most critical neurological disorders [1]. Epilepsy manifests as unpredictable and repetitive seizures that disrupt regular brain functioning. Mortality due to epilepsy is a major concern throughout the world, and epileptic patients are at a higher risk of death than the general population by 2- to 3-fold [2]. Most patients with epilepsy have additionally been reported to have one or more behavioural or cognitive comorbidities, including depression and memory decline [3]. During epileptogenesis, deviations take place at different levels, including membrane channels, intracellular signaling cascades, synaptic connectivity, and genes, to elicit an enduring state of hyperexcitability and hypersynchrony. Neurotransmitter alteration is likewise one of the main culprits underlying seizure pathophysiology [4].

An intriguing connection between autophagy and epilepsy has arisen, as defective autophagy has been shown to enhance epilepsy [5]. The process of autophagy involves the formation of double-membrane vesicles called autophagosomes, which subsequently fuse with lysosomes to degrade cytoplasmic components and pathogens and maintain cell homeostasis [6]. Since autophagy is associated with the control of synaptic transmission and plasticity; excitotoxicity; neurodegeneration; astroglial death; as well as axon, synaptic and mitochondrial function, it is possible that aberrant autophagy could enhance anomalous axon plasticity, synaptic remodelling and, ultimately, the formation of epileptic networks. In this context, autophagy seems to be an attractive therapeutic target for epilepsy [7].

Although antiepileptic drugs (AEDs) are the backbone of treatment, nearly one-third of patients are noncompliant with such pharmacological interventions. Currently accessible alternatives such as deep brain or vagal nerve stimulation, ketogenic diet and surgery are either not applicable to all patients or only partly effective [8,9]. The restorative capability of stem cells affords a promising therapeutic avenue for disorders of the central nervous system (CNS) by virtue of their multi-differentiability, culture expansibility, insignificant immunogenicity, and other features [10]. Moreover, these cells are relatively safe to use in humans [11]. Endothelial progenitor cells (EPCs), normally originating from endothelial stem cells, are able to differentiate into endothelial cells that are vital for neovascularization. The functional role of circulating EPCs is to sustain vascular integrity [12].

Epileptic seizures significantly affect the neurovascular unit. This pathologic neurovascular response may cause a drop in the energy supply, thereby enhancing cellular damage and decelerating energy-requiring homeostatic processes, for example, the function of active transporters required for neuronal repolarization. These alterations eventually extend the depolarization state and delay seizure termination [13]. Although EPCs are crucial in the management of many vascular insult-linked diseases, they have not yet been investigated in epilepsy. Accordingly, the present study aimed to investigate, for the first time, the potential therapeutic effects of EPCs in a pentylenetetrazole (PTZ)-induced kindling model of epilepsy. Kindling features progressively increasing convulsion activity owing to the repeated application of electrical or chemical sub-convulsive triggers [14]. Chemical kindling seizures triggered by PTZ are assumed to mimic the pathogenesis of human epilepsy and are considered a model of drug-resistant epilepsy [15].

Material and methods

Animals

Male Wistar albino rats weighing 170 ± 30 g, obtained from the animal facility of the Faculty of Pharmacy, Cairo University, were used in the current study. The rats were housed in standard plastic cages under controlled temperature (25 ± 2 °C) and illumination (12 h light/12 h dark) conditions and had access to a pelleted standard rat chow diet and *ad libitum* water. Rats were left for one week as an initial adaptation period before any experimental manipulation. *The study was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised in 1978) and was approved by the Ethical Committee of Animal Care and Use of Faculty of Pharmacy, Cairo University (Approval Number: BC 1838).*

Drugs and chemicals

Pentylenetetrazole (PTZ) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Valproic acid (Depakine®) was procured from Sanofi-Aventis (Paris, France). All other chemicals were of pure analytical grade.

Experimental design

Rats were randomly divided into four experimental groups of 15 rats each. Group I (the normal control group) consisted of rats that received only vehicle. Group II (the PTZ group, kindled rats) comprised rats that were subjected to intraperitoneal injections of a sub-convulsive dose of PTZ (35 mg/kg) 3 times/week [9] with a total of 9 injections, where three consecutive generalized convulsions were achieved by this regimen (stage 5 on Racine's scale; falling and status epilepticus) [16]) as shown in Suppl. Fig. 1. Group III (the EPCs group) comprised kindled rats that received a single intravenous dose of EPCs (2×10^6 cells) injected in the rat tail vein after the first generalized convulsion was recorded [17]. Group IV (the VPA group) consisted of kindled rats that received valproic acid orally (150 mg/kg) as a reference antiepileptic drug 30 min before each PTZ injection.

Separation and culture of EPCs

Bone marrow was aspirated from the femora and tibiae of male syngeneic Fisher-344 rats (150–200 g). Rats were obtained from the farm of the National Institute for vaccination, Helwan, Egypt. Animals were housed in polycarbonate cage for a 2-week acclimation period. Purified rodent diet and water were allowed *ad libitum* to all rats. A 12-h light/dark cycle was maintained during the entire procedure. Temperature and humidity were maintained between 23 and 25 °C and 40–60%, respectively. Density gradient centrifugation was performed using Ficoll-Paque PLUS (Amersham, Buckinghamshire, England) at 400*g* for 30 min to isolate the mononuclear cells (MNCs). After centrifugation, the cells were re-suspended in culture medium containing 20% foetal bovine serum, 50 μg/mL streptomycin, 50 U/mL penicillin, 2 mmol/L glutamine,

5 ng/mL basic fibroblast growth factor, 50 ng/mL VEGF (Gibco/BRL, NY, USA), then cultured on fibronectin-coated flasks and incubated for 7 days at 37 °C in 5% CO_2 to obtain EPCs. The culture medium was replaced every 48 h [17]. Flow cytometric immunophenotyping (Beckman Coulter, EPICS-XL, Atlanta, Georgia, USA) was used to characterize the EPCs cluster of differentiation (CD31, CD34 and CD133) [18].

Labelling EPCs with PKH26

EPCs were harvested and then labelled with the stable red fluorochrome PKH26 (Sigma-Aldrich Chemical Co.), which has an excitation wavelength of 551 nm and an emission wavelength of 567 nm. The biological and proliferative properties of PKH26-labelled cells are retained; thus, PKH26-labelled cells are perfect for *in vivo* tracking. After 2 washes in serum-free medium, the cells were pelleted, suspended in a PKH26 solution, and eventually injected intravenously in the tail veins of the rats [17].

Fluorescence imaging

EPCs were kept in acetylated low-density lipoprotein (LDL, 10 μg/mL) (Molecular Probes, Eugene, OR, USA) at 37 °C for 2 h for *in vitro* fluorescent staining. Afterwards, the cells were fixed for 10 min in 2% paraformaldehyde. Fluorescence imaging of the rat hippocampus was conducted using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) to confirm the homing of the injected cells [19].

Sampling

At the end of the experimental period, rats were sacrificed by decapitation under light anaesthesia 24 h after neurobehavioural assessment. The brains were rapidly isolated, and the hippocampi were harvested and divided into two portions. The first portion was weighed and homogenized in ice-cold lysis buffer, pH 7.4, containing 25 mM HEPES; 0.1% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; 5 mM $MgCl_2$; 1.3 mM EDTA; 1 mM EGTA; 10 μg/mL pepstatin, aprotinin, and leupeptin; and 1 mM phenylmethanesulfonyl fluoride (PMSF). The resulting homogenate was used to estimate hippocampal GABA, glutamate, serotonin, dopamine and acetylcholine levels. The second portion was homogenized on ice using a Polytron handheld homogenizer (Thomas Scientific, NJ, USA) in a lysis buffer, pH 7.4, containing 50 mM Tris-HCl, 10 mM NaF, 2 mM EDTA, 1 mM PMSF and 10 mM β-glycerol phosphate and supplemented with an EDTA-free protease inhibitor cocktail. The resulting brain homogenates were used for determination of hippocampal markers of autophagy, namely, light chain protein-3 (LC-3), beclin-1 and autophagy-related gene-7 (ATG-7).

Brain samples from each group were kept in 10% buffered formol saline for 24 h. These specimens were used for subsequent fluorescence imaging, histopathological examination and immunohistochemical characterization of brain-derived neurotrophic factor (BDNF).

Seizure severity score

After each PTZ injection, all animals were watched for 30 min to determine the seizure severity score. This score was evaluated utilizing Racine's scale [16]. Racine's scale depicts 6 stages: stage 0; normal non-epileptic activity, stage 1; snout and facial movements, hyperactivity, grooming, sniffing, scratching, and wet dog shakes, stage 2; head nodding, staring, and tremor, stage 3; forelimb clonus and forelimb extension, stage 4; rearing and salivating, and stage 5; falling and status epilepticus.

Neurobehavioural assessment

Y-maze spontaneous alternation test

This test was performed in a Y-maze that consisted of 3 identical arms labelled A, B and C, each with dimensions of 40 cm (length) × 35 cm (height) × 12 cm (width), positioned at equal angles [20]. Animals were put at the end of one arm and were allowed to move freely throughout the maze for a 5 min period. The pattern of entries into each arm was observed for each animal. When a rat's hind paws were situated entirely on an arm, arm entry was considered complete. Consecutive entrances into the maze's three arms in overlapping triplet sets were regarded as alternation. Same-arm return (SAR) scores were documented. The total number of alternations and total number of arm entries were also documented, from which the spontaneous alternation percentage (SAP) was computed as the number of alternations divided by the total number of possible alternations (i.e., the total number of arm entries minus 2) and multiplied by 100.

Open field test (OFT)

The OFT was conducted in a 113 × 113 × 44 cm chamber with wooden walls and a Plexiglas floor. The floor was coated black with white lines that created a 5 × 5 grid pattern. The rat was placed in one corner of the chamber and monitored for 3 min. Every time the rat crossed a single line from one grid square into a nearby square with the four paws, the event was recorded to calculate ambulation frequency. Other behavioural patterns were also measured as latency time, grooming (frequency with which the animal licked or scratched itself while stationary) and rearing (frequency with which the rat stood on its hind legs in the field).

Determination of hippocampal neurotransmitters (GABA, glutamate, serotonin, dopamine, and acetylcholine)

Commercially available ELISA kits were used for determination of GABA (EIAab Ltd., Wuhan, China), glutamate (MyBioSource, San Diego, USA), serotonin (LifeSpan BioSciences, Inc. Seattle, WA, USA), dopamine (Cusabio, Wuhan, China) and acetylcholine (Cusabio, Wuhan, China). All procedures conformed strictly to the manufacturer's guidelines.

Western blotting analysis of hippocampal LC-3, beclin-1 and ATG-7 protein expression

Homogenization of hippocampal samples was carried out using a lysis buffer containing a protease inhibitor cocktail (Bio Basic Inc., Markham, ON, Canada) as previously described. After centrifugation (15,000*g*, 30 min at 4 °C), the supernatant was assayed for protein concentration using a Bradford protein assay kit SK3041 (Bio Basic Inc., Markham ON, Canada). Then, aliquots of 50 μg of protein were isolated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Roche Diagnostics, IN, USA). The membranes were blocked at 37 °C for 1 h using 5% non-fat dried milk dissolved in Tris-buffered saline with TWEEN 20 (TBST), then incubated overnight at 4 °C with specific primary antibodies against LC-3, beclin-1, ATG-7 and β-actin (Thermo Fisher Scientific, Waltham, USA) diluted 1:2000 in PBS. Thereafter, horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) were applied at a dilution of 1:3000 for 1 h at 37 °C. Each treatment was followed by 3–5 washes with 0.1% TWEEN in TBST (0.01 M, pH 7.6). Subsequently, the resulting bands were identified using an enhanced chemiluminescence (ECL) assay (Pierce Chemical Co., Rockford, USA) and an Alliance 4.7 Gel documentation system (UVITEC, UK) according to the manufacturer's protocol. The band densities obtained by Western blotting analysis

were estimated with UV Tec software (England, UK) [17,21]. The band intensities were normalized to β-actin.

Histopathological studies

Haematoxylin and eosin (H&E)-stained, deparaffinized sections (3 μm thick) were observed through a light microscope equipped with a built-in camera (Leica Microsystems, Wetzlar, Germany). A scoring system was used to evaluate the degree of severity of the histopathological alterations; where 0 indicated none, 1 indicated mild (changes <30%), 2 indicated moderate (changes 30–50%), and 3 referred to severe (changes >50%) [22].

Immunohistochemical detection of hippocampal BDNF

Deparaffinized sections were subjected to microwave antigen retrieval for 5 min before peroxidase quenching with 3% H_2O_2 in PBS for 15 min. Next, the sections were pre-blocked for 30 min with 5% bovine serum albumin and incubated overnight at 4 °C with primary antibodies specific to BDNF (GeneTex Inc., CA, USA). The sections were washed with PBS, incubated for 30 min with biotinylated secondary antibody and stained with 3,3′-diaminobenzidine (DAB) solution (Dako, Glostrup, Denmark). Finally, the slides were counterstained with haematoxylin for 2–3 min and examined using a digital video camera installed on a Leica DMLB2 light microscope (Leica Microsystems, Wetzlar, Germany). The fractions of brown DAB-positive immunoreactive area were then quantified.

Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM). Differences among groups were statistically tested by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc multiple comparisons test. Data of Y-maze test, OFT (grooming, rearing and ambulation frequency) and % of BDNF-positive cells were presented as the median (interquartile range) and analysed using a non-parametric Kruskal–Wallis test followed by Dunn's post-test for multiple comparisons. All statistical analyses were conducted using GraphPad Prism version 6.0 (GraphPad® Software, San Diego, California, USA). Statistical significance was assumed at $P < 0.05$.

Results

Characterization and recognition of fluorescent signals from EPCs in the rat hippocampus

EPCs metabolic activity was confirmed via uptake of acetylated LDL (Suppl. Fig. 2). EPCs were characterized by the surface expression of cluster of differentiation (CD31, 34 and 133) markers using flow cytometry. The percentages of their expression were 30%, 65% and 36%, respectively (Fig. 1a). The migration of EPCs to the hippocampus of PTZ-kindled rats was investigated by means of fluorescence imaging. PKH26-labelled EPCs were recognized in the brains of epileptic animals, confirming their successful homing (Fig. 1b).

Effect of EPCs on the seizure severity score

Rats treated with PTZ progressively experienced Racine's stages from almost no behavioural manifestation to major tonic-clonic convulsions. On the other hand, the rats treated with VPA exhibited a significant reduction in Racine's score when compared to the epileptic one. Likewise, injection of EPCs ensued a significant reduction in the seizure severity score in comparison with the PTZ-group. Noteworthy, stem cells induced a significant improvement in this score compared to VPA (Suppl. Fig. 2).

Effect of EPCs on PTZ-induced changes in neurobehavioural function

The effect of EPCs on spatial memory was tested by evaluating spontaneous alternation behaviour through the Y-maze test. PTZ administration significantly decreased the SAP and augmented the SAR scores relative to those of the control group. These actions were significantly counteracted via the administration of EPCs achieving significantly higher SAP and lower SAR scores compared to those of the VPA group. However, the total number of arm entries showed no significant changes between the studied groups (Fig. 2a–c).

The motor activity of epileptic rats in the OFT was significantly decreased. The epileptic group had a longer latency time than the control group to exhibit motor activity, while the EPCs and VPA groups showed significantly reduced latency time; this effect was more pronounced in the EPCs group than in the VPA group (Fig. 2d). Moreover, all rats treated with PTZ exhibited motor impairment (grooming and rearing) in comparison to control rats. Ambulation frequency was significantly lower in the PTZ group than in the control group. Treatment with either EPCs or VPA enhanced motor coordination, as manifested by increased ambulation frequency. The EPCs group exhibited a significant enhancement in motor coordination (grooming, rearing and ambulation frequency) compared to the VPA group (Fig. 2e–g).

Effect of EPCs on PTZ-induced changes in hippocampal neurotransmitters

As shown in Fig. 3, administration of PTZ significantly lowered the hippocampal γ-aminobutyric acid (GABA), serotonin, dopamine and acetylcholine (ACH) levels by 84%, 74%, 67% and 80%, respectively, compared to those of the control group and caused a concomitant 223% elevation in the glutamate level. On the other hand, EPCs countered these changes in epileptic rats in a manner analogous to VPA. However, EPCs afforded a significantly more pronounced elevation in GABA and dopamine levels than VPA provided.

Effect of EPCs on PTZ-induced changes in hippocampal protein expression of autophagy markers

As depicted in Fig. 4, induction of epilepsy significantly suppressed the hippocampal protein levels of LC-3, beclin-1 and ATG-7 by nearly 69%, 82%, and 75%, respectively, compared with the corresponding control values as revealed by Western blotting analysis.

The administration of EPCs elicited significant 3-fold, 5-fold and 4-fold increases, respectively, in the protein levels of the studied autophagy markers compared to the levels in the PTZ group, with effective normalization of beclin-1 and ATG-7 expression. Similarly, the VPA group showed significantly elevated protein levels, with no significant difference from the EPCs group.

Effect of EPCs on histopathological alterations

Normal control brain sections showed intact hippocampal structure (Cornu Ammonis, CA part) in which the neurons had the typical histological form (Fig. 5a). In contrast, in the PTZ group, the hippocampal neurons showed degeneration along with severe necrosis and vacuolization (Fig. 5b and c).

Sections from the VPA group revealed attenuated pathological changes, although areas of necrosis and slight vacuolation of some

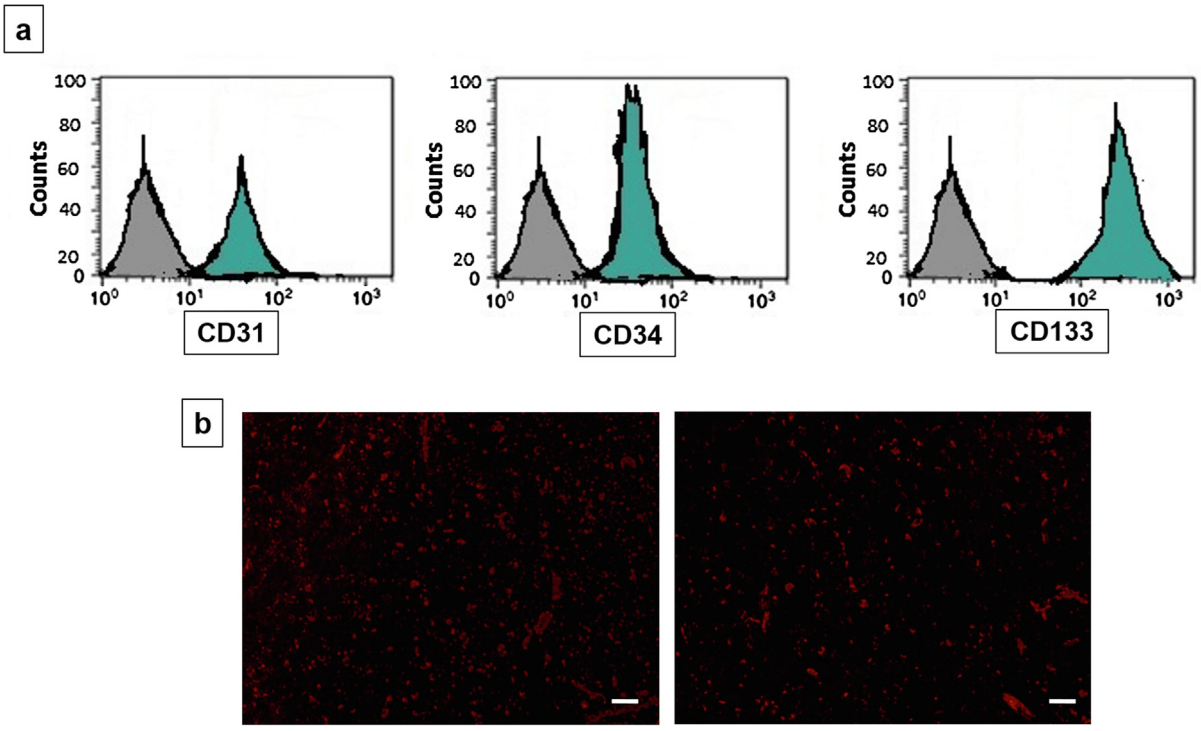


Fig. 1. Characterization and recognition of fluorescent signals from EPCs in the rat hippocampus. a) A flow cytometric assay showed the presence of CD31, 34, and 133 surface markers on EPCs engrafted to the hippocampus after their administration. b) Homing of EPCs to the hippocampus of rats was verified through recognition of red fluorescent signals from PKH26-marked cells (scale bar 100 μm). EPCs, endothelial progenitor cells.

hippocampal neurons were still noticed (Fig. 5d and e). Administration of EPCs mitigated these histological alterations, giving the hippocampal area an almost normal histological structure comparable to that of the control group (Fig. 5f).

Effect of EPCs on PTZ-induced changes in immunohistochemical staining of hippocampal BDNF

The data in Fig. 6 indicate that PTZ administration significantly reduced the level of BDNF in the hippocampus area by 46% compared with that of the control group, as demonstrated by evaluating the percentages of immunostained area. However, EPCs markedly enhanced the levels of BDNF to 161% compared to the corresponding PTZ group values, thereby restoring normal expression levels. Notably, the outcome achieved by EPCs was significantly more prominent than that obtained by VPA.

Discussion

The inadequacies of present treatment for refractory epilepsy have provided an impetus to consider alternative therapeutic strategies, including stem cell therapy [23]. To the best of our knowledge, this study is the first demonstration of the therapeutic potential of EPCs in a model of epilepsy.

Based on their proliferative, differentiative and regenerative capacities, stem cells could reinstate neural circuits and restore the physiological excitability of neurons lost during the disease [24]. The administration of EPCs in the present study significantly reduced glutamate and elevated GABA levels, thus correcting the delicate excitation-inhibition balance. In fact, PTZ-induced kindling might be connected to permanently curbing the inhibitory capacity of the brain GABAergic system, chiefly through inhibition of $GABA_A$ activated channels. In addition, PTZ alters the sensitivity and density of various glutamate receptor subtypes, thereby increasing the density of glutamate in the hippocampus [14].

The hippocampus has been recognized for its role in learning and memory processes. Therefore, hippocampal damage following kindled seizures may cause cognitive impairment [25]. The epileptic animals in this study demonstrated memory and behavioural perturbations, as manifested by their depressed performance in the Y-maze test and the OFT, compared to control animals. These findings are consistent with other studies indicating behavioural changes and long-term memory deficits accompanying the PTZ-induced kindling process [9,26]. Promisingly, administration of EPCs caused improvements not only in seizure severity but also in motor incoordination and cognitive impairment associated with epilepsy. This behavioural and cognitive modulatory capability of EPCs grants them an advantage over many AEDs, such as phenytoin, carbamazepine and valproate that can modify the progression of kindling but not the related depression and memory decline [27].

In the present study, the observed behavioural changes in epileptic animals were well correlated with neurochemical alterations, as demonstrated by the reduced brain dopaminergic, serotonergic and cholinergic levels compared to the control animals. Such altered neurochemical status is consistent with previous reports [28,29] and might account for the observed seizure threshold reduction, memory impairment and behavioural alterations in PTZ-treated animals. Dopamine has been shown to curb the abnormal neuronal hyperexcitability associated with the kindling process [30]. Furthermore, depletion of norepinephrine and serotonin has been shown to exacerbate neuronal damage and seizures in humans with epilepsy as well as animal models of epilepsy [4,31]. Moreover, there is broad consensus on the role of acetylcholine in learning and memory [32]. In the present study, EPCs administration effectively restored hippocampal dopamine, serotonin and acetylcholine levels in rats with epilepsy, thereby helping mitigate the severity of their seizures and improve their cognitive and behavioural status.

Neuronal death caused by seizures results mainly from excitotoxicity and increased glutamatergic transmission with

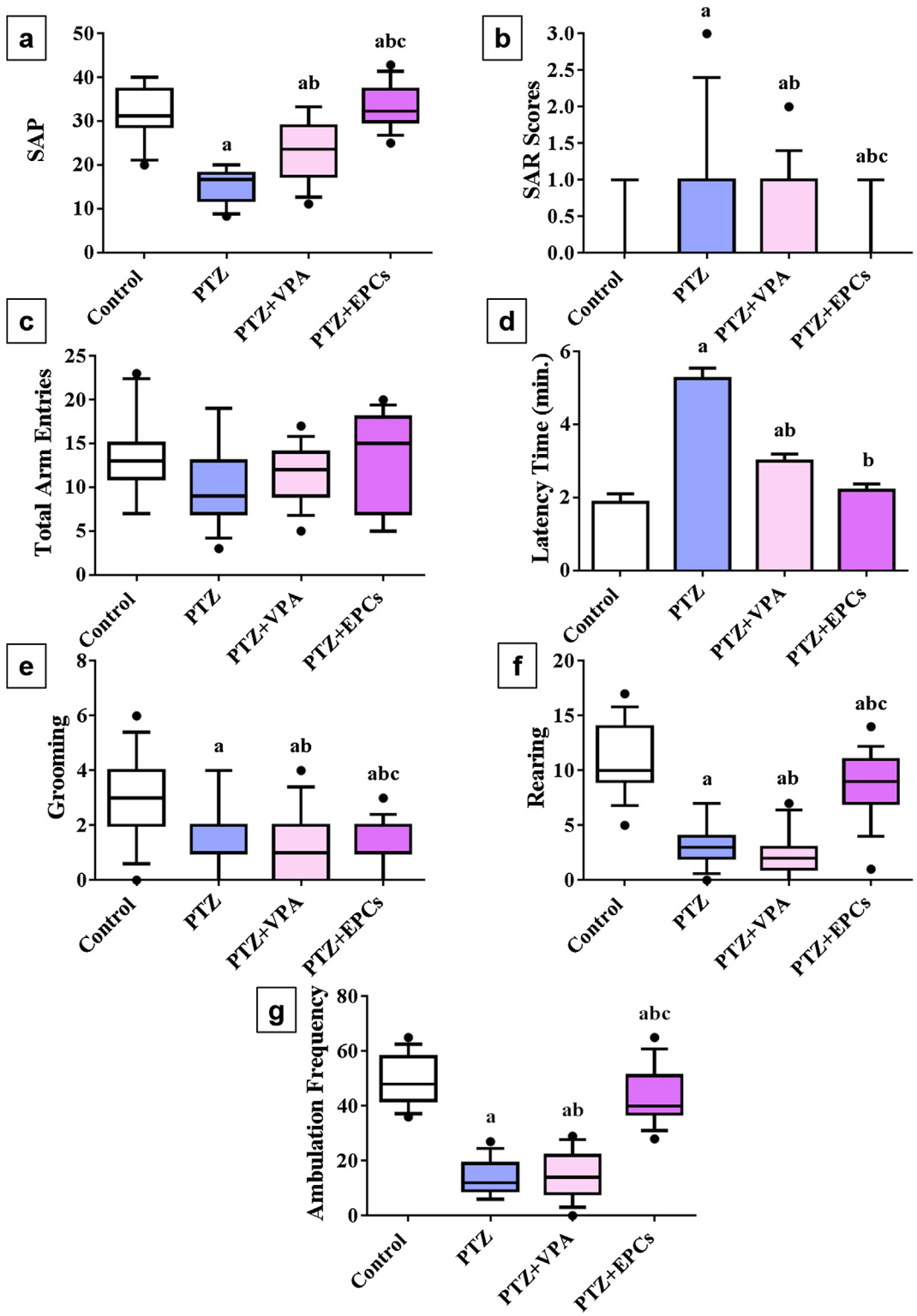


Fig. 2. Effect of EPCs on PTZ-induced changes in neurobehavioural function: Y-maze test (a–c) and OFT (d–g). Each horizontal line inside the box plots of SAP (a), SAR scores (b), total arm entries (c), grooming (e), rearing, and (f) ambulation frequency, and (g) represents the median; the boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the intervals between the 10th and 90th percentiles. Filled circles indicate data points outside the 10th and 90th percentiles. These parameters were analysed using the Kruskal–Wallis test followed by Dunn's post-test for multiple comparisons. Latency time (d), for which each column with a vertical line represents the mean ± SEM, was analysed using one-way ANOVA followed by Tukey-Kramer test. [a] significantly different from the control group, [b] significantly different from the PTZ group, [c] significantly different from the VPA group at $P < 0.05$. PTZ, pentylenetetrazole; VPA, valproic acid; EPCs, endothelial progenitor cells; SAP, spontaneous alternation percentage; SAR, same-arm returns.

subsequent DNA damage and protease activation, eventually leading to necrosis, although apoptotic activation has also been reported [33]. This understanding is consistent with the histopathological observations depicted herein, which reveal severe neuronal necrosis and vacuolation in the hippocampus of epileptic rats. The benefits of stem cell transplantation are

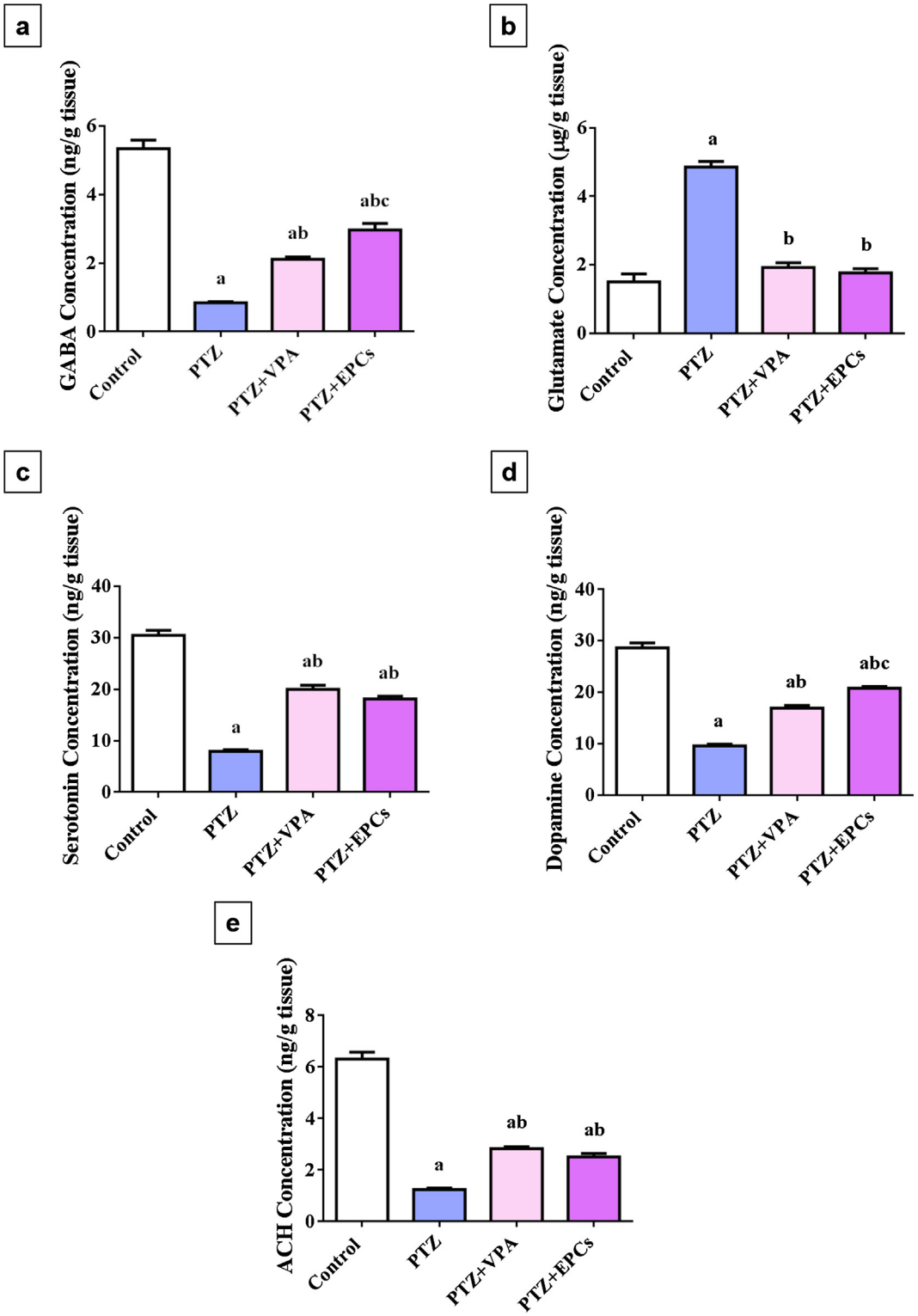


Fig. 3. Effect of EPCs on PTZ-induced changes in hippocampal neurotransmitters. GABA (a), glutamate (b), serotonin (c), dopamine (d), and ACH (e) concentrations. Each column with a vertical line represents the mean ± SEM. [a] significantly different from the control group, [b] significantly different from the PTZ group, and [c] significantly different from the VPA group at $P < 0.05$ using one-way ANOVA followed by a Tukey-Kramer test. PTZ, pentylenetetrazole; VPA, valproic acid; EPCs, endothelial progenitor cells; GABA, γ-aminobutyric acid; ACH, acetylcholine.

attributed to replacing the degenerated neurons, conferring an improved milieu for the injured tissue, sparing the rest of the neurons and hindering inflammation by releasing chemokines and growth factors to enhance cell survival and endogenous recovery [34]. In the present study, EPCs reversed all histopathological changes caused by PTZ administration and led to an almost normal hippocampal structure. Thus, it could be speculated that EPCs have the ability to regenerate neuron populations and compensate for the neuronal death associated with epilepsy.

Cell survival, proliferation and differentiation in the CNS are controlled by a large group of growth factors. BDNF in particular is reportedly involved in epileptogenic processes [35]. The current

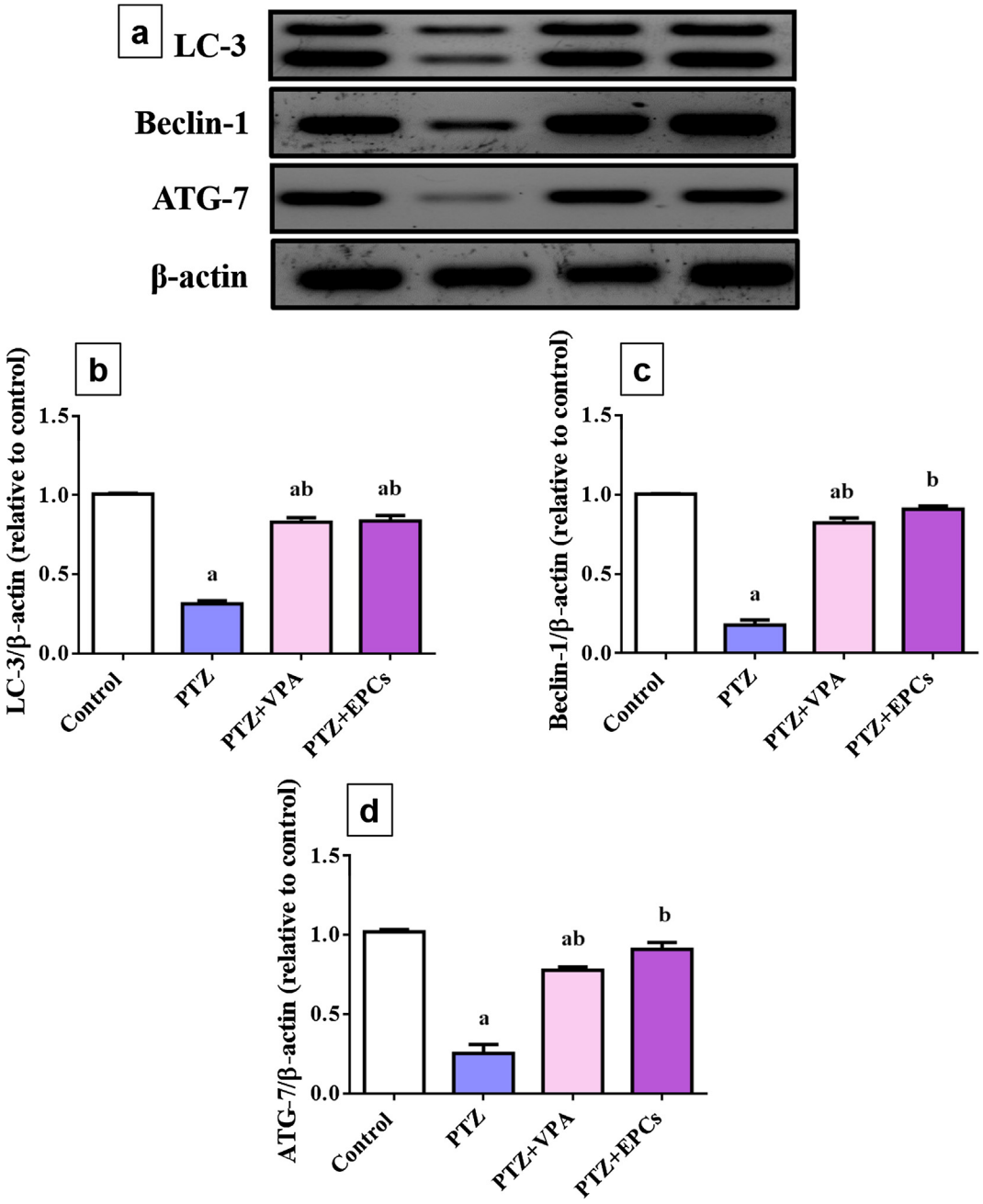
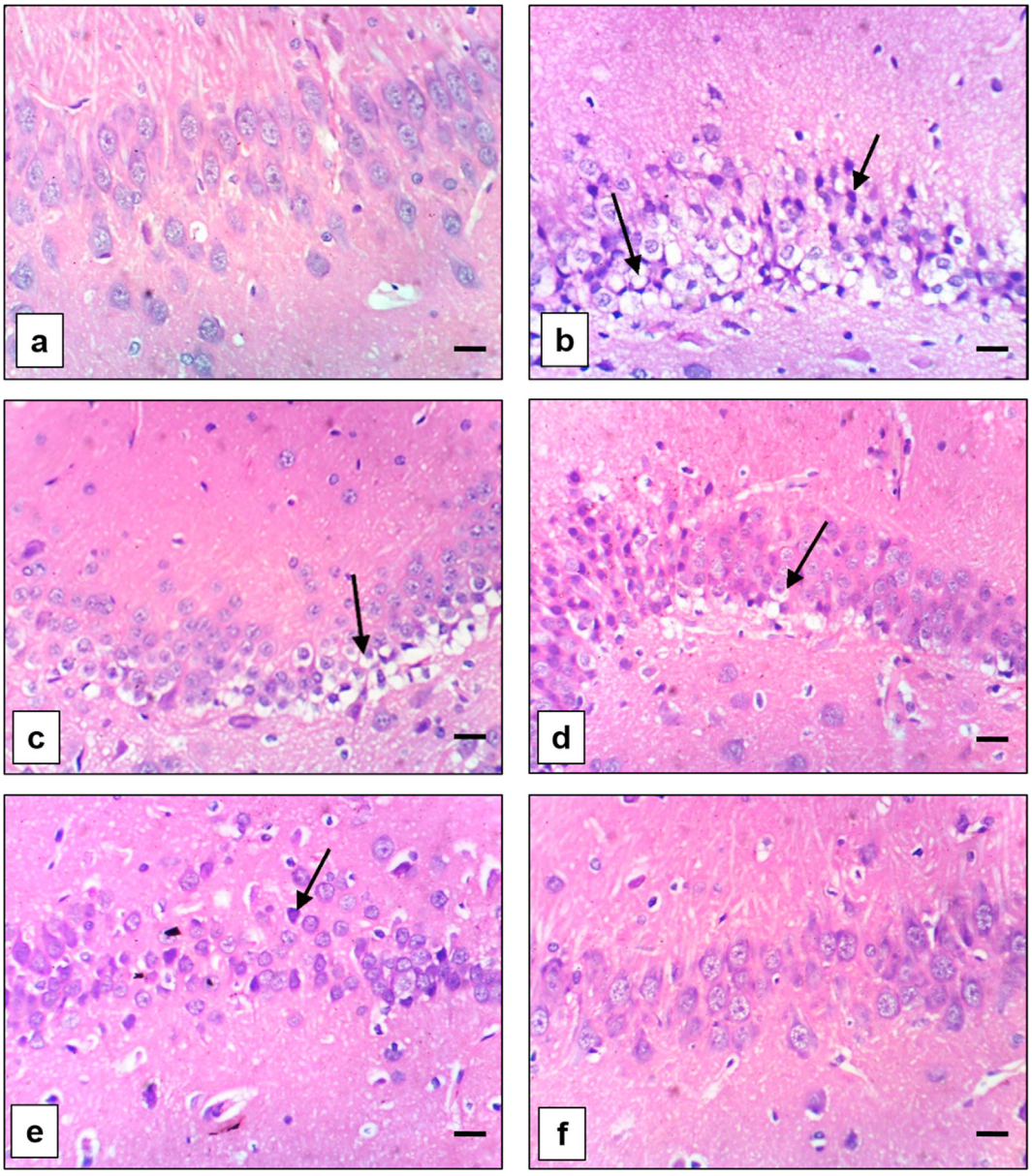


Fig. 4. Effect of EPCs on PTZ-induced changes in hippocampal protein expression of the autophagy markers LC-3, beclin-1 and ATG-7. Band densities obtained by Western blotting analysis were quantified and normalized to those of β-actin (a). Each column with a vertical line of LC-3 (b), beclin-1 (c), and ATG-7 (d) represents the mean ± SEM. [a] significantly different from the control group, [b] significantly different from the PTZ group, and [c] significantly different from the VPA group at $P < 0.05$ using one-way ANOVA followed by a Tukey-Kramer test. PTZ, pentylenetetrazole; VPA, valproic acid; EPCs, endothelial progenitor cells; LC-3, light chain protein-3; ATG-7, autophagy-related gene-7.

study revealed decreased expression of BDNF in the brain tissue of epileptic rats as detected by immunohistochemistry. The hippocampal region shows the highest plenitude of BDNF, which is a key determinant of neuronal differentiation, development, and protection against diverse insults [17]. BDNF is also crucial for dopaminergic, glutamatergic and serotonergic neurotransmission. Reduced hippocampal BDNF levels were reported to dysregulate long-term potentiation, which is a cellular form of synaptic plasticity associated with learning and memory, and to disrupt the establishment and consolidation of hippocampus-dependent memory in rats [36].

Accumulating evidence indicates that EPCs release growth factors such as BDNF that provide defence against axonal degeneration and enhance the survival of neurons [37,38]. Indeed, the present findings indicate that epileptic tissues exhibit a significant elevation in BDNF levels in response to EPCs administration, suggesting that this mechanism could contribute to the concomitant preservation of cognition. Thus, the transplanted cells could exert a paracrine effect and supply the epileptic brain with certain neurotrophic factors.

Cellular quality control depends on two degradation pathways for the disposal and recycling of cellular garbage. One pathway is the ubiquitin-proteasome system, which specifically degrades ubiquitin-labelled proteins. The other pathway is the autophagy-lysosomal system, which accomplishes bulk degradation and recycling of non-functional proteins and organelles [39]. In the brain,



Histopathological lesion	Control	PTZ	VPA	EPCs
Vacuolation of neurons	0	3	2/1	0
Necrosis of hippocampus pyramidal neurons	0	3	1	0

Fig. 5. Effect of EPCs on histopathological alterations. a) Control group displaying normal histological structure of the hippocampus. b and c) PTZ-treated rats showing severe neuronal necrosis with vacuolation in the hippocampus (arrow). d and e) PTZ + VPA group displaying mild neuronal necrosis and vacuolation (arrow) of the hippocampus. f: PTZ + EPCs-treated group demonstrating mitigated histopathological alterations with almost normal hippocampal structure. PTZ, pentylenetetrazole; VPA, valproic acid; EPCs, endothelial progenitor cells (scale bar 100 μm). (0) no histopathological changes, (1) mild changes, (2) moderate changes, (3) severe changes.

autophagy has mainly been considered with regards to the clearance of aggregates, and thereby hampering the neurodegenerative process [40].

Several investigations ascribe the occurrence of epilepsy as a consequence of defective autophagy to the deficiency of ATG-18 [41] or ATG-7 [42]. The loss of ATG-7, a key player in the autophagy cascade, in a transgenic mouse model leads to spontaneous seizures, implying that suppression of autophagy is sufficient to induce epilepsy [6]. In support of the role of autophagy disruption in the pathogenesis of epilepsy, the present findings revealed perturbations of autophagy marker levels, as manifested by suppression of LC-3, beclin-1 and ATG-7 protein expression, in the PTZ-treated group.

It is worth mentioning that impeded autophagy could trigger the occurrence of epilepsy, and, conversely, epilepsy could likewise bring about the dysregulation of autophagy, which could further exacerbate epilepsy, creating a vicious cycle [5]. Autophagy is a catabolic process that liberates free amino acids through protein degradation. Since amino acids act as neurotransmitters or precursors of neurotransmitters, their metabolic homeostasis in the brain is critical. Consequently, a failure of autophagy could disturb the homeostasis of neurotransmitters that are implicated in brain

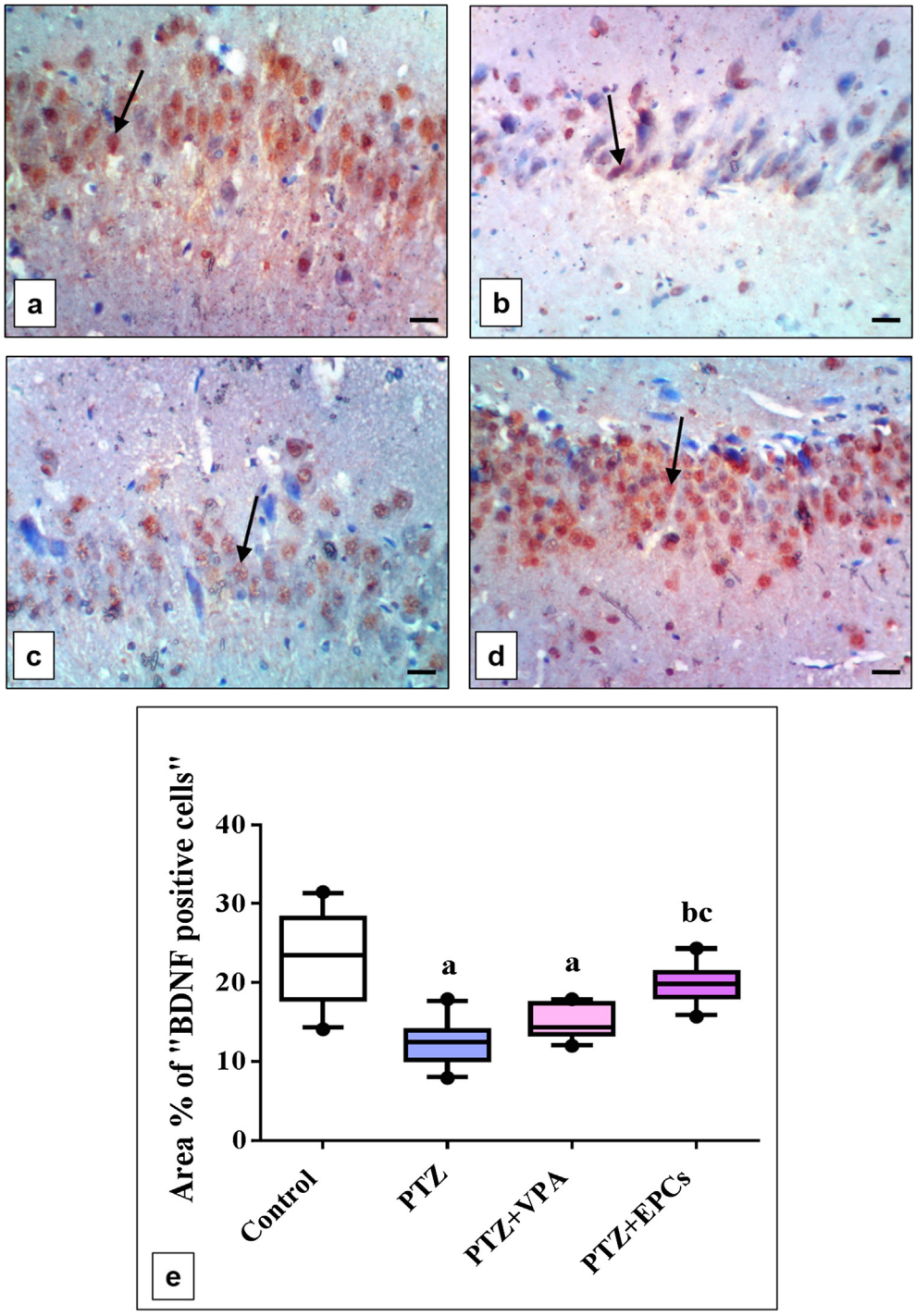


Fig. 6. Effect of EPCs on PTZ-induced changes in immunohistochemical staining of hippocampal BDNF. a) Control group. b) PTZ-treated group. c) PTZ + VPA group. d) PTZ + EPCs group. e) Quantification of BDNF (calculated as the area of BDNF-immunopositive cells as a percentage of the total area of the microscopic field across ten fields). Horizontal lines inside the box plots represent the median; the boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the intervals between the 10th and 90th percentiles. Filled circles indicate data points outside the 10th and 90th percentiles. [a] significantly different from the control group, [b] significantly different from the PTZ group, and [c] significantly different from the VPA group at $P < 0.05$ using the Kruskal–Wallis test followed by Dunn's post-test for multiple comparisons. PTZ, pentylenetetrazole; VPA, valproic acid; EPCs, endothelial progenitor cells; BDNF, brain-derived neurotrophic factor (scale bar 100 μm).

physiology and pathophysiology [43,44]. In light of this justification, the observed autophagic alterations might explain, at least in part, the corresponding changes in neurotransmitter levels. Promisingly, therapeutic approaches that invigorate autophagy are utilized for the treatment of certain epileptic disorders [44]. In the present study, EPCs provoked a significant increase in LC-3, beclin–1 and ATG-7 protein expression levels, supporting their potential therapeutic effects in epilepsy. Interestingly, this study has investigated the role of EPCs in ameliorating epilepsy-associated abnormalities, suggesting autophagy upregulation as a possible underlying mechanism. However, our findings do not rule out the possibility that the enhanced autophagy might be an accompanying event rather than a causal factor of the amendments achieved by EPCs treatment. Hence, further research using

autophagy-deficient animal models is necessary to verify the autophagy promoting capacity of EPCs and to better elucidate the potential causal relationship between the anti-epileptic ability of EPCs and autophagy upregulation.

From the findings of the present study, it is obvious that treatment with EPCs not only achieved comparable effects to VPA regarding correcting neurotransmitter levels and autophagy protein levels but also achieved superior results regarding behavioural and cognitive modulation, reversing the histopathological changes and their effects on BDNF levels.

Conclusions

The current study is the first to demonstrate the potent therapeutic efficacy of EPCs in a rat model of epilepsy, in which they amend the behavioural, biochemical, vascular and histological alterations associated with epilepsy. The underlying mechanisms may involve, at least in part, the upregulation of autophagy. Promising results regarding the safe and beneficial use of EPCs have been revealed in initial clinical trials in patients with certain pathologies. However, the successful use of EPCs therapy in neurological disorders is still limited with very few reports in animal models. We herein accentuate an efficient anti-epileptic potential of EPCs, providing a promising treatment strategy that could reduce the disease burden and improve the quality of life in the epilepsy patient population. Therefore, we urge further research in this area to enhance the feasibility of EPCs-based treatment of epilepsy and to surmount the drawback of insufficient quantities of EPCs that can be generated from patients.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jare.2019.01.013.

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