Original Article

Asian Pacific Journal of Tropical Biomedicine

apjtb.org

doi: 10.4103/2221-1691.389573

Impact Factor® 1.7

Ocimum sanctum extract preserves neuronal echotexture and controls seizure in lithium-pilocarpine induced status epilepticus rats

Soumya Sucharita Pattnaik[1], Sudhir Chandra Sarangi[1]✉, Deeksha Sharma[1], Surabhi Sinha[1], Tapas C Nag[2], Aruna Nambirajan[3], Manjari Tripathi[4]

[1]*Department of Pharmacology, All India Institute of Medical Sciences, New Delhi, India*

[2]*Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India*

[3]*Department of Pathology, All India Institute of Medical Sciences, New Delhi, India*

[4]*Department of Neurology, All India Institute of Medical Sciences, New Delhi, India*

ABSTRACT

Objective: To investigate the effect of *Ocimum sanctum* hydroalcoholic extract (OSHE) on seizure control and neuronal injury in rats with lithium-pilocarpine-induced status epilepticus (SE).

Methods: SE was induced by administering lithium chloride followed by pilocarpine 24 h later. OSHE was administered either alone or in combination with valproate (VPA) 3 days before SE induction until 14 days post-SE induction. Seizure parameters were recorded on day 1 (0-3 h), day 1-3 and day 4-14 post-SE. On day 14 post-SE, neurobehavioural tests (elevated plus maze and passive avoidance) were done followed by total antioxidant capacity, neuron-specific enolase, immunohistochemistry, and electron microscopic assessment in the hippocampus and cortex tissue.

Results: OSHE+VPA provided more significant seizure protection (75%) than VPA (62.5%), OSHE (62.5%), or SE control (12.5%) (overall P=0.003). The latency to stage-3/4 seizures was increased and the number of stage-3/4 seizures was reduced in all treatment groups compared to the SE control group (P=0.002 and <0.001, respectively). The OSHE+VPA group also had better memory retention than other treatment groups (P<0.001) in the passive avoidance test. Total antioxidant capacity level was significantly higher and neuron-specific enolase was lower in the OSHE and OSHE+VPA groups compared to the SE control group. Electron microscopic study showed significant myelin sheath damage (67.5%, P<0.05) and axonal degeneration (51.8%, P<0.001) in the hippocampus of the SE control group, which were alleviated by OSHE or OSHE+VPA treatment. In immunohistochemical analysis, the OSHE, OSHE+VPA, and VPA groups had a significantly higher number of viable neurons and less neuronal loss compared to the SE control in the hippocampus (P<0.001).

Conclusions: OSHE either alone or in combination with VPA shows better seizure control by preservation of neuronal echotexture and reducing oxidative stress in the hippocampus.

KEYWORDS: Status epilepticus; *Ocimum sanctum*; Seizure protection; Neuronal injury; Electron microscopy

Significance

Ocimum sanctum has shown anticonvulsant, antioxidant, and neuroprotective effects in acute and chronic experimental seizure models; however, there is scarce evidence regarding its potential in the treatment of status epilepticus. This study reveals that *Ocimum sanctum* showed better seizure control and neuroprotective effects either alone or in combination with valproate.

✉To whom correspondence may be addressed. E-mail: scsarangi@gmail.com

How to cite this article: Pattnaik SS, Sarangi SC, Sharma D, Sinha S, Nag TC, Nambirajan A, et al. *Ocimum sanctum* extract preserves neuronal echotexture and controls seizure in lithium-pilocarpine induced status epilepticus rats. Asian Pac J Trop Biomed 2023; 13(11): 479-487.

Article history: Received 30 June 2023; Revision 2 September 2023; Accepted 10 November 2023; Available online 24 November 2023

1. Introduction

Status epilepticus (SE) is a neurological emergency that needs immediate management due to its considerable mortality and morbidity. It can affect individuals of all ages[1]. The annual incidence of SE is 10-41 per 100 000 people, with a substantial mortality rate of more than 20%[2]. A significant proportion of patients do not respond to first- and second-line treatments and require more aggressive management in the intensive care unit[3]. There is alteration in neuronal network and neuronal injury following SE which may result in long-term consequences such as neurodegeneration and cognitive dysfunctions. SE is a condition caused by either a failure of the mechanisms responsible for the termination of seizure or the activation of mechanisms that result in abnormally prolonged seizures. A common characteristic feature of SE broadly includes the development of epileptic foci and injury primarily in the limbic region, followed by a latency period, during which an epileptogenic process takes place, which in turn leads to the development of spontaneous recurrent seizures, *i.e.*, the chronic epileptic phase. In addition to seizures, many patients after SE suffer from behavioral alterations and impairment of learning and memory, which appear to be progressive over time[4].

The animal model of SE for screening of anti-seizure medications (ASM) is the most widely used approach for inducing chronic epilepsy, especially temporal lobe epilepsy (TLE). Unlike in acute seizure models, animals in TLE models have spontaneous recurrent seizures without any provocation. The SE episode serves as a trigger for initiation of the epileptogenesis in TLE. TLE is the most common form of drug-resistant epilepsy which affects nearly 20% of all patients with epilepsy[5].

Ocimum sanctum (*O. sanctum*) L. commonly known as "Tulsi" in India has shown anticonvulsant as well as neuroprotective effects in animal models of acute and chronic seizures. In our previous study, *Ocimum per se* showed 50% protection in the maximal electro-shock seizures model; moreover, its combination with ASM carbamazepine and phenytoin enhanced the seizure protection potential. It also exerted better memory retention potential in combination with ASMs as evident from the elevated plus maze (EPM) and Morris-water maze test[6]. When *O. sanctum* was given alone or in combination with levetiracetam in the pentylenetetrazole kindling model of epilepsy, better seizure control, and neuroprotection as well as reduced oxidative stress were observed[7]. In another study, *Ocimum per se* showed anticonvulsant potential, and its combination with valproate improved neurobehavioural function and reduced oxidative stress[8]. In addition, no significant pharmacokinetic interaction was observed when *O. sanctum* was administered with standard ASMs such as valproate, carbamazepine, phenytoin, and levetiracetam.

Though *Ocimum* has demonstrated anticonvulsant and neuroprotective effects in various animal models of acute and chronic seizures, evidence is scarce regarding the adjuvant potential of *Ocimum* in the SE model. Hence, this study aimed to investigate the role of *O. sanctum* in combination with standard ASM valproate (VPA) in lithium-pilocarpine-induced SE rat model and its sequalae-like neuronal injury.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (200-250 g) were kept in polyacrylic cages under standard laboratory conditions with a natural light and dark cycle (12 h:12 h). They were allowed to access a standard pellet diet and tap water *ad libitum*. The experiment was started after 7 days of acclimatization.

2.2. Plant material used in this study

The leaves of *O. sanctum* L. (botanical name/synonym: *Ocimum tenuiflorum* L.; family: Lamiaceae; common name: 'Tulsi' in India) were used to acquire the hydroalcoholic extracts (OSHE) (batch no. FOS2107012, July-2021) used in this study. The botanical name of this plant has been checked with http://www.theplantlist.org. This was obtained as a free-gift sample from Natural Remedies Pvt. Ltd., Bangalore, India. According to the certificate of analysis (Supplementary Figure) issued by the quality control department of the same manufacturer, OSHE contained ursolic acid and oleanolic acid 2.6%, rosmarinic acid 0.4%, and eugenol 0.5% *w/w* (by HPLC).

2.3. Chemicals and reagents

Pilocarpine hydrochloride was obtained from TCI Co Ltd., India; lithium chloride (LiCl) anhydrous and dimethyl sulfoxide (DMSO) from SRL Pvt. Ltd. India; glycopyrrolate from Neon Laboratory Ltd. India and VPA tablet from Sanofi Healthcare India Pvt. Ltd. India.

2.4. Experimental design

The rats were randomly assigned into five groups (n=8) as follows: vehicle-control (received 10% DMSO as the vehicle), SE control (10% DMSO), VPA (370 mg/kg), OSHE (1 000 mg/kg), VPA+OSHE. The experimental design is presented in Figure 1.

2.5. Dosage of test drugs and route of administration

The dose of VPA was determined in a previous experimental study based on the maximum human recommended dose of 370 mg/kg[8]. OSHE at a dose of 1 000 mg/kg was used in this study as this dose has shown optimum protection against seizures in both acute and chronic models of epilepsy in rats. In our previous study, a dose range of 200, 400, 800, and 1 000 mg/kg was administered for 14 d to screen the optimal dose for seizure protection. OSHE at doses of both 800 and 1 000 mg/kg showed seizure protection in 50% of rats in both the maximal electro-shock and pentylenetetrazol

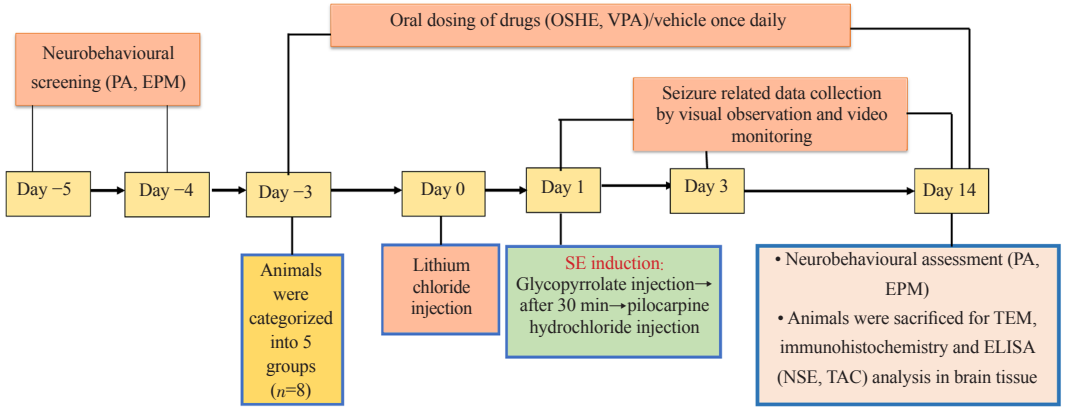

Figure 1. Experimental design. OSHE: *Ocimum sanctum* hydroalcoholic extract; VPA: valproate; PA: passive avoidance; EPM: elevated plus maze; SE: status epilepticus; TEM: transmission electron microscopy; ELISA: enzyme-linked immuno sorbent assay; TAC: total antioxidant capacity; NSE: neuron-specific enolase.

models of acute seizures. At 1 000 mg/kg, there was increased latency and decreased duration of seizures as compared to 800 mg/kg. Moreover, this dose showed better memory retention potential in neurobehavioural tests either alone or in combination with VPA[8]. Based on these facts, 1 000 mg/kg was selected as the optimum dose to be used in this study. All the test drugs were dissolved in 10% DMSO (vehicle) and administered once daily orally from day −3 (3 days before induction of SE) to 14 days post-induction of SE. On the day of SE induction, test drugs were administered 30 min before pilocarpine administration.

2.6. Induction of SE

The seizure score was assessed as per the scale previously used in our study[7], which is categorized as no response (score 0), ear and facial twitching (score 1), head nodding and myoclonic body jerks (score 2), forelimb clonus (score 3), and generalized tonic-clonic seizures with loss of righting reflex or hind limb tonic extension (score 4). Peak seizure behavior was considered to correspond to a score of 3 or 4, *i.e.*, stage-3/4 seizure.

The SE induction was done in accordance with the Reduced Intensity Status Epilepticus model with a little modification[9]. SE was induced by administration of LiCl (127 mg/kg *i.p.*) followed by pilocarpine hydrochloride (25 mg/kg *i.p.*) administration 24 h later. Glycopyrrolate was administered 30 min before pilocarpine injection to avoid its peripheral cholinergic signs. If the animal did not show stage-3/4 seizures, a 2nd dose of pilocarpine 25 mg/kg after 1 h of the 1st dose and a 3rd dose of 15 mg/kg after 1 h of the 2nd dose were re-administered. Hence, a maximum of 3 doses of pilocarpine (25 mg/kg, 25 mg/kg, and 15 mg/kg) were administered. Even after these 3 doses, if the animals did not exhibit seizures, that was considered as the protective effect of the drugs used in the study or resistance of the animal. The SE induction was performed in the morning session (around 8:00 a.m.) to avoid diurnal variation in seizure.

The animals were observed by both continuous video-monitoring and visual observation by keeping them in individual transparent cages. Animals were observed visually by two individual observers for an initial 3 h followed by at least 6 h (3 h in the morning and 3 h in the evening) per day till 14 days post-SE. The video monitoring was later analyzed to find out any missed seizure episodes. The following parameters were recorded post-SE induction: a. Latency to stage-3/4 seizures, b. % of rats with stage-3/4 seizures, c. number of stage-3/4 seizures, d. mortality.

2.7. Neurobehavioural tests

EPM and passive avoidance (PA) tests were performed two days before the administration of test drugs (day −4 and day −5) for screening of animals and also on day 14 post-SE induction as described in our previous study[6].

2.8. Brain tissue collection for immunohistochemistry (IHC) and transmission electron microscopy (TEM)

Brain tissue was collected at the end of the neurobehavioral assessment. On day 14 post-SE, the rats of all groups were euthanized with high-dose pentobarbital, and the whole brain was carefully removed from the cranial cavity. The blood was then removed from the isolated brain by washing it in cold saline (0.9% sodium chloride). The brain was made into two halves (through sagittal suture) and the hindbrain parts were excluded. One-half of the brain cortical tissue was used for oxidative stress assessment and from the same half the hippocampus tissue was isolated for electron microscopy assessment. The other half was examined for IHC. For oxidative stress, the tissue was immediately washed in ice-cold 0.1 M sodium phosphate buffer saline and stored at −80 ℃. For IHC, tissue was kept in 10% formalin, and for electron microscopy assessment, tissues were kept in the fixatives (compositions: 2.5% glutaraldehyde, 1% paraformaldehyde, 0.1 M phosphate buffer

saline, and 1% osmium tetraoxide) available for this analysis.

2.9. TEM

The brain samples were taken out, and placed on a cold plate and the hippocampus was immediately isolated after separating it from the cortex. Hippocampus tissue was fixed in fixative. The samples then were dehydrated in cold acetone and embedded in Araldite CY2 12. Ultrathin sections (60-70 nm) were cut using Ultracut UC7, and stained with uranyl acetate and lead citrate. The grids were washed with distilled water and viewed under a transmission electron microscope (TALOS 200X, G2 HR TEM; Thermo Fisher Scientific, Netherlands) at a primary magnification of 2 250 ×.

2.10. IHC for detection of glial fibrillary acid protein (GFAP) and anti-neuronal nuclear protein (NeuN) in brain tissues

Immunohistochemical staining was used to analyze the treatment efficacy by determining anti-NeuN a marker for mature/viable neurons and GFAP an established marker for reactive astrocytes.

Half of the brains postfixed in 10% formalin overnight were grossed by taking coronal sections of 3-4 mm thickness and placing them in tissue cassettes. The tissue was processed in an automated histoprocessor followed by embedding into a paraffin block. From each block, three serial sections were taken on glass slides: one stained for microscopic examination using hematoxylin and eosin stain (H&E) and two unstained sections taken on poly-lysine coated slides for IHC.

Under light microscopy of the H&E-stained slides, the dorsal hippocampus was identified. Areas of infarcts and inflammation were noted. For IHC, the coated slides were dewaxed by washing them as follows: three times in xylene solution for 5 min, once in acetone for 3 min, once in 95% ethanol for 3 min, and then rehydrating in distilled water (5-7 min). After that, antigen retrieval was done using 0.01 M citric acid (pH 6.0) and was kept at 100 ℃ for 30 min followed by immediate one wash with water when the temperature was cooled down to room temperature. After three washes with 0.1 M Tris (hydroxymethyl)aminomethane buffer for 5 min, the sections were incubated in 4% H_2O_2 for 10 min to eliminate the endogenous peroxidase activity (tyramide signal amplification). The protein blocker was used for 3 min to prevent non-specific antibody attachment to tissue. GFAP (1∶500; mouse monoclonal, Invitrogen) and NeuN (1∶1 000; Rabbit monoclonal, Invitrogen) were chosen as the primary antibodies and were maintained overnight in a humid environment at 4 ℃. Following overnight incubation, sections were washed three times with Tris buffer solution for 5 min at room temperature, followed by a 10-min incubation with the primary antibody amplifier and three more times with 0.1 M Tris buffer. It was then developed in 3,3-diaminobenzidine (DAB, 1-2 min), rinsed three times with 0.1 M Tris buffer (5 min each), treated with avidin-biotin horseradish peroxidase complex for 1 h, and then incubated with 0.1 M Tris buffer to halt the reaction. All of the sections underwent hematoxylin counterstaining, three rounds of washing in 0.1 M Tris buffer, drying, and cover-slipping.

2.11. Quantification of NeuN and GFAP expression

In this study, the CA1, CA2, CA3, and CA4 regions of the dorsal hippocampus (4 areas) and frontal cerebral cortex regions (1 area) were photographed at 100× magnification using a light microscope (BX53, Olympus, Japan). On the photomicrographs, the number of neurons stained with NeuN (brown nuclear-stained cells) and the number of reactive glial cells (brown stained star-shaped astrocytes) were manually tagged and counted using DotDotGoose software (version 1.5.3.).

2.12. Total antioxidant capacity (TAC) and neuron-specific enolase (NSE) estimation by ELISA

Brain cortical tissues were taken in ice-cold 0.1 M phosphate buffer saline with pH 7.4 and 10% homogenate was made. Homogenates were centrifuged at 10 000 ×g for 10 min at 4 ℃ to isolate the supernatants, which were then kept at −80 ℃.

TAC and NSE were evaluated in homogenates of the cortical tissues as per the instructions of the ELISA kit manufacturer (GenAsia Biotech Co. Ltd., Shanghai). The absorbance was measured at 450 nm using an ELISA microplate reader (MB-580, BR Biochem Life Sciences Pvt. Ltd., India). A calibration curve was made by taking the above-mentioned standards and the concentration of the above parameters in the samples was calculated from the obtained equation from the graph.

2.13. Statistical analysis

The data were checked for normality (skewness and kurtosis) and represented as mean ± SD (for normally distributed data) or as median (range) (for non-normally distributed data) for continuous variables and as a percentage for categorical variables. For normally distributed data, one-way ANOVA followed by Bonferroni *post-hoc* test was done to compare between more than two groups, and Kruskal-Wallis's test with multiple comparisons was done to compare between more than two groups for non-normally distributed data. The IBM SPSS Statistics 23.0 software was used to analyze the data. A *P*-value less than 0.05 was considered statistically significant.

2.14. Ethical statement

The experimental procedure was approved by the Institutional Animal Ethics Committee (approval no. 274/IAEC-1/2021, dated 30.06.2021) and conducted in accordance with 'The Committee for the Control and Supervision of Experiments on Animals' guidelines, Department of Animal Welfare, Government of India.

3. Results

3.1. Effect of O. sanctum on seizure parameters in SE rats

Rats of the SE control group had significantly lower latency to stage-3/4 seizures as compared to other drug-treated groups (overall P=0.011). More significantly higher latency was observed in VPA and VPA+OSHE group, compared to SE control (P=0.043, and 0.017, respectively). Seizure protection (% of rats without stage 3/4 seizures) was higher in the OSHE+VPA group (75%) than in other groups (VPA-62.5%, OSHE-62.5%, SE control-12.5%) on day 1 to 3. No mortality was observed in the OSHE and VPA+OSHE groups whereas it was 25% and 12.5% in the SE control and VPA group, respectively. All drug-treated groups had a significantly lower number of stage-3/4 seizures compared to the SE control group on day 1 (0 to 3 h) and from day 1 to 3; whereas, only the SE control group showed stage-3/4 seizures from day 4 to 14 (Table 1).

3.2. Effect of O. sanctum on neurobehavioural parameters

In the EPM test, the drug-treated groups did not show any significant decrease in transfer latency compared to the SE control group. However, memory retention potential was significantly improved in all drug-treated groups in terms of increased retention latency in PA as compared to the SE control group (P<0.001). Rats treated with both OSHE and VPA showed better memory as evidenced by significantly increased retention latency [(270.8 ± 19.5) s] compared to the VPA [(193.3 ± 11.5) s] or OSHE alone treated group [(187.8 ± 21.3) s] (P<0.001 for both groups) and it was similar to that of the vehicle control group [(291.6 ± 20.4) s] (Figure 2A and 2B).

3.3. Effect of O. sanctum on TAC and NSE levels

TAC level was significantly higher in the OSHE and OSHE+VPA groups [(19.6 ± 2.7) U/mL and (21.7 ± 3.1) U/mL, respectively] as compared to the SE control group [(9.7 ± 1.4) U/mL, P=0.035 and 0.008, respectively], which signifies the antioxidant potential of *O. sanctum*. The combination of OSHE with VPA exhibited markedly higher TAC levels as compared to VPA treatment alone. In addition, rats in the SE control group had a significantly higher level of NSE [(29.17 ± 5.12) ng/mL] compared to all drug-treated groups [VPA: (10.49 ± 1.67) ng/mL, OSHE: (11.33 ± 4.05) ng/mL and OSHE+VPA: (9.28 ± 1.30) ng/mL, P<0.001]. NSE levels did not differ significantly among the drug-treated groups (Figure 2C and 2D).

3.4. Effect of O. sanctum on electron microscopic changes in brain tissues

Rats in the SE control group had significantly higher mean percentage myelin sheath damage (67.5%, P<0.05) and axonal degeneration (51.8%, P<0.001) as compared to other drug-treated groups. The VPA group had lower mean percentage degenerated myelin sheath (14.1%) and axon (15.8%) compared to other drug treated groups. The OSHE+VPA had lesser damaged myelin sheath and axonal degeneration compared to the group treated with OSHE alone though it was not found to be statistically significant. The

Table 1. Effect of *Ocimum sanctum* on seizure parameters in status epilepticus rats.

Parameters	SE control	VPA	OSHE	OSHE+VPA
Latency to stage-3/4 seizure (min) [median (range)]	73 (39-180)	180 (106-180)$^{\#}$	180 (120-180)	180 (124-180)$^{\#}$
% Mortality (no. of rats)	25 (2/8)	12.5 (1/8)	-	-
No. of stage- 3/4 seizure [median (range)]				
Day 1: 0-3 h	5.5 (0-9)	0 (0-1)$^{\#}$	0 (0-5)$^{\#}$	0 (0-4)$^{\#}$
Day 1 to 3	7 (2-10)	0 (0-2)$^{\#}$	0 (0-5)$^{\#}$	0 (0-4)$^{\#}$
Day 4 to 14	7	-	-	-
% with stage-3/4 seizure (no. of rats)				
Day 1 to 3	87.5 (7/8)	37.5 (3/8)	37.5 (3/8)	25 (2/8)
Day 4 to 14	83.33 (5/6)	-	-	-

VPA: valproate, OSHE: *Ocimum sanctum* hydroalcoholic extract, SE: status epilepticus. $^{\#}$indicates a significant difference compared to the SE control.

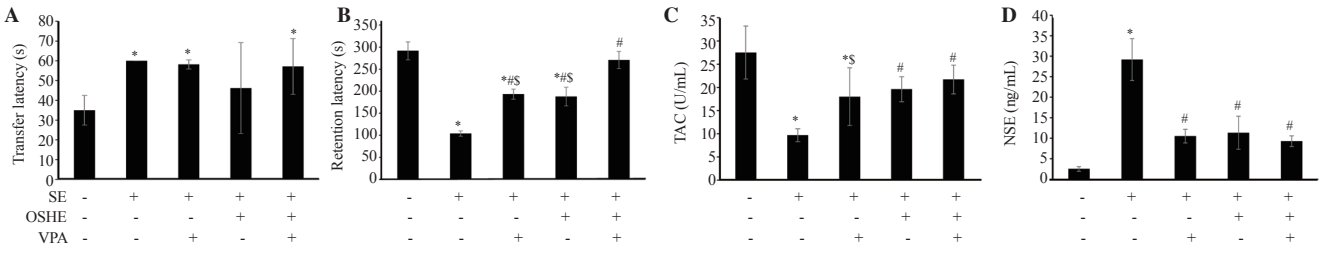

Figure 2. Effect of *Ocimum sanctum* on neurobehavioural parameters in elevated plus maze (A), and passive avoidance test (B), as well as total antioxidant capacity (TAC) (C), and neuron-specific enolase (NSE) (D). The data are expressed as mean± SD (n=8 in vehicle control, OSHE and OSHE+VPA groups; n=7 in the VPA group; n=6 in the SE control group). $^{*}P$<0.05 *vs.* vehicle control, $^{\#}P$<0.05 *vs.* SE control, and $^{\$}P$<0.05 *vs.* OSHE+VPA.

severity of nuclear changes (nuclear condensation and shrinking) and cytoplasmic changes (increased numbers of vacuoles and disorganization of cytoplasmic organelles) was more pronounced in the SE control group, which were reduced in all treatment groups (Figure 3).

3.5. Effect of O. sanctum on IHC assessment of hippocampus and cerebral cortex

In the IHC assessment, the neuronal marker NeuN staining was done to characterize the curative effect of drugs in SE-induced neuronal loss/damage. IHC analysis revealed that SE control rats had extensive neuronal loss in the CA1, CA2, CA3, and CA4 regions of the hippocampus compared to the vehicle control and drug-treated groups. Similarly, significant neuronal damage was found in the cerebral cortex of the SE control group compared to others (P<0.001). All treatments significantly increased the number of viable neurons and alleviated neuronal loss in the CA2, CA3, and CA4 regions of the hippocampus (P<0.001). The OSHE+VPA group had significantly reduced neuronal loss in the CA1 region compared to the SE control (P=0.004). Furthermore, all drug-treated groups showed a significantly increased number of viable neurons in the cortex compared to the SE control group (P<0.001) (Figure 4).

Antibodies against GFAP were used to identify reactive astrocytosis present in the hippocampus and cortex of epileptic rats. Quantification of GFAP positive stain revealed that rats in the SE control group had significantly higher reactive astrocytes compared to the vehicle control and other drug-treated groups both in the hippocampus as well as the cortex (all P<0.001). Among drug-treated groups, VPA had a fewer number of reactive astrocytes in hippocampus compared to the OSHE (P=0.001) and OSHE+VPA groups (though not significant). However, no significant difference was found in cortex astrocyte count when a comparison was done between the drug-treated groups (Figure 4).

4. Discussion

The current study investigated the adjuvant role of *O. sanctum* in seizure control, neurobehavioral parameters, and neuronal injury in lithium-pilocarpine-induced SE rats. We found that *O. sanctum* either alone or in combination with VPA showed significant seizure control and memory retention, as well as reduced oxidative stress

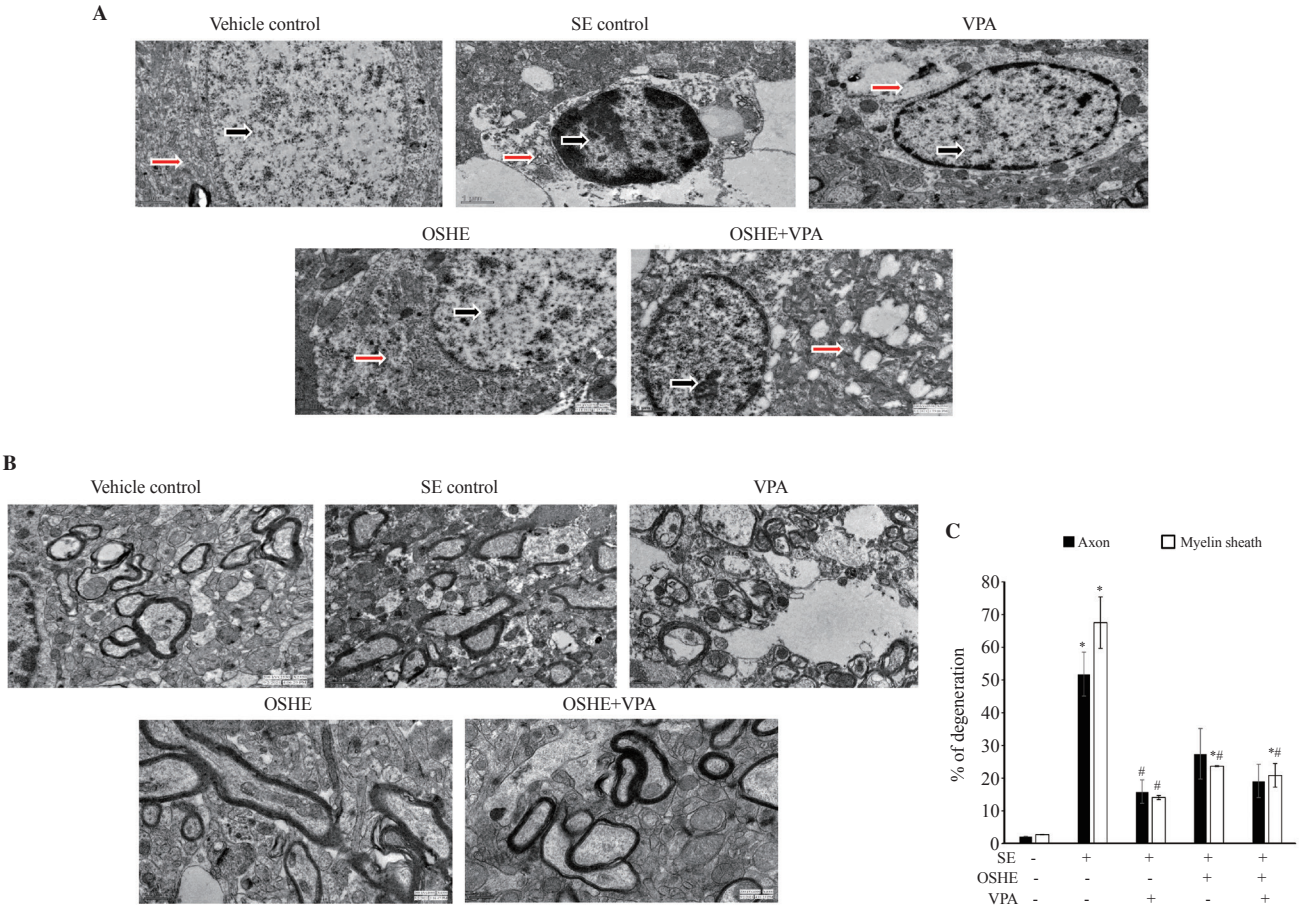

Figure 3. Transmission electron microscopic images showing (A) nuclear and cytoplasmic changes, as well as (B) axon and myelin sheath changes in the hippocampus (n=3 in each group) (magnification: 2250×, scale bar: 1 μm). The black arrow shows the change in nuclear chromatin; the red arrow shows the change in cytoplasmic organelles. (C) A bar graph shows % of axon and myelin sheath degeneration. $^{*}P$<0.05 *vs.* vehicle control, $^{\#}P$<0.05 *vs.* SE control (analyzed by ANOVA followed by Bonferroni *post-hoc* test).

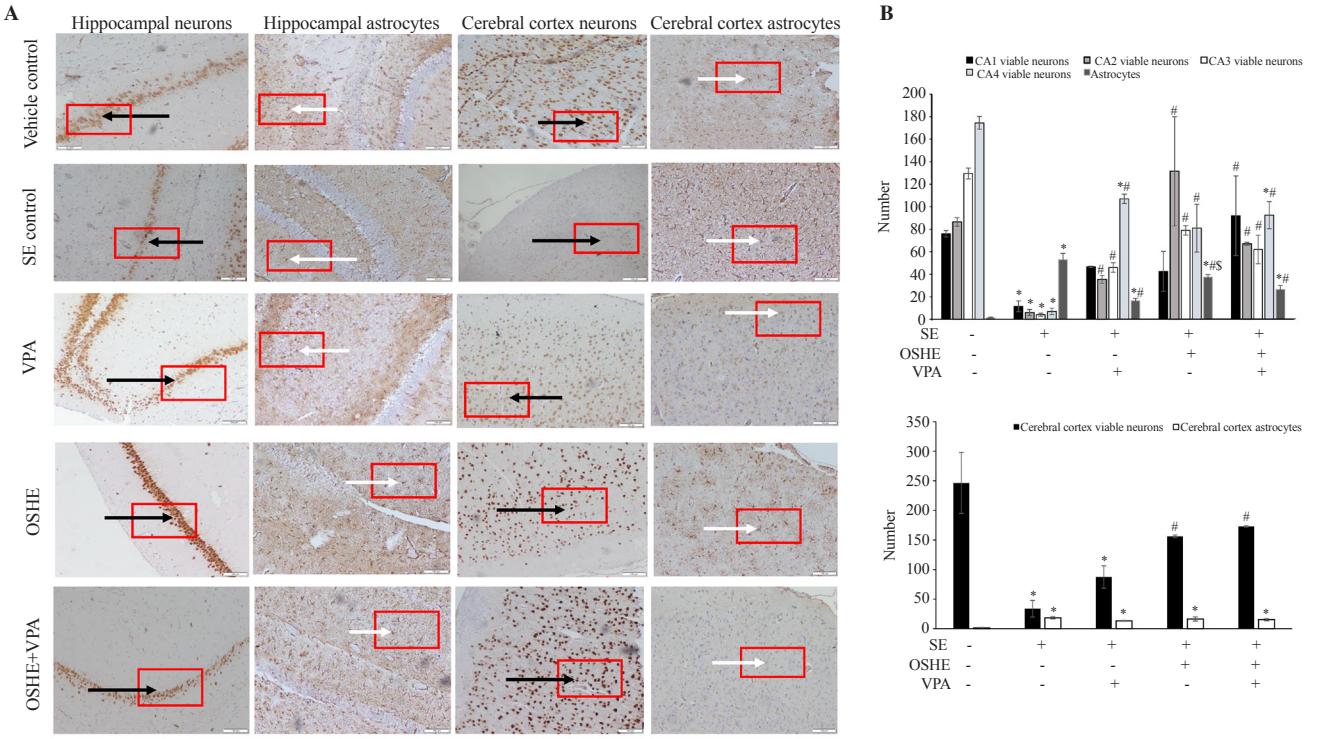

Figure 4. (A) Immunohistochemical changes showing NeuN-immuno-stained dorsal hippocampus (CA1) and cortical neurons, and GFAP-immuno-stained hippocampal and cortical astrocytes (n=3 in each group) (magnification: 100×, scale bar: 100 μm). The black arrow shows neurons whereas the white arrow shows astrocytes. (B) In the graphs, the number of viable neurons or astrocytes in the hippocampus and cerebral cortex is shown. $^{*}P$<0.05 *vs.* vehicle control, $^{\#}P$<0.05 *vs.* SE control, and $^{\$}P$<0.05 *vs.* VPA.

and neuronal loss compared to the SE control group.

The primary goal of SE treatment is to end epileptic activity as soon as possible, ideally before t2, *i.e.*, 30 min following the occurrence of SE to prevent long-term consequences such as neuronal injury and cognitive dysfunction[10]. Hence, in patients with a high risk of developing SE in later stages (*e.g.*, patients with acquired brain insults), novel therapies that could reduce the risk of SE and attenuate its sequelae need to be explored. There should be minimal pharmacokinetic interactions of these agents with ongoing ASMs. In this context, OSHE which has shown significant seizure control activity and minimal interactions with ASMs like VPA, levetiracetam, carbamazepine, and phenytoin in previous studies may have a role in early prophylactic treatment for individuals with a high risk of developing SE.

VPA is the second-line treatment of choice for the management of SE along with other ASMs such as phenytoin, fosphenytoin, phenobarbitone, and levetiracetam. It has a wide spectrum of efficacy with multiple mechanisms of action. Some of the studies found VPA to be as effective as phenytoin. In a study, VPA prevented seizures in 66%-88% of patients with SE or acute repetitive seizures[11]. A meta-analysis considering the pooled data from eight studies found that VPA showed 75.7% efficacy in aborting SE which is the highest efficacy of any of the medications reviewed[12]. VPA has been also shown to have anti-epileptogenic and neuroprotective properties by some epigenetic mechanisms such as histone deacetylase modulation[13]. Taking these facts into consideration, we used VPA as the standard ASM in this study.

In the current study, *O. sanctum* in combination with VPA had comparable efficacy with VPA in terms of increased latency to stage-3/4 seizures. Moreover, the seizure protection was significantly higher in the group treated with *O. sanctum* alone as well as in the combination group as compared to the SE control. In previous studies, *O. sanctum* has shown variable effects in seizure control with different ASMs. In one of these studies, administration of *O. sanctum per se* for 14 d led to 50% protection in maximal electroshock-induced seizures and its combination with ASMs such as carbamazepine and phenytoin enhanced the seizure protection potential[6]. In another study, chronic administration of *O. sanctum* in combination with levetiracetam led to better seizure control as compared to levetiracetam alone in the kindling model of epilepsy[7]. However, administration of *O. sanctum* with VPA did not enhance its antiepileptic potential in the acute seizure model as compared to VPA alone[8].

In a recent study, it has been reported that treatment with ursolic acid, an active ingredient of OSHE, markedly repressed SE-induced neuroinflammation by decreasing inflammatory mediators such as IL-1β and TNF-α. Moreover, oxidative stress markers were significantly downregulated following ursolic acid administration, which suggests its anti-inflammatory and antioxidant potential. Ursolic acid also reduced aberrant neurogenesis, hippocampal neuronal loss, and ectopic migration, indicating its neuroprotective potential[14]. Taking these facts into consideration, it may be

postulated that ursolic acid, a major constituent of *O. sanctum* may potentially contribute to its anticonvulsant action by suppressing neuroinflammation and oxidative stress induced by epilepsy.

In this study, *O. sanctum* in combination with VPA showed better memory retention potential as compared to VPA or *O. sanctum* alone as evident in the PA test. This is in agreement with a recent study in which administration of *O. sanctum* extract either alone or in combination with levetiracetam reversed the memory impairment in amyloid beta-induced Alzheimer's disease model in rats. Furthermore, these treatments restored the hippocampal architecture by inhibiting the neuronal loss in the CA1, CA3, and dentate gyrus as well as decreased the neuronal excitotoxicity by decreasing glutamate and increasing γ-aminobutyric acid levels. *O. sanctum* also demonstrated antioxidant activity in this study[15]. In our previous study, a combination of OSHE with VPA resulted in better memory retention potential compared to the VPA-alone treated group[8]. In another study, OSHE alone or in combination with carbamazepine and phenytoin improved memory as compared to disease control in neurobehavioural tests (PA and Morris-water-maze). These facts signify that *O. sanctum* may have the potential to improve cognition in epilepsy[6].

Various experimental studies have shown that oxidative stress results from the activation of inducible nitric oxide synthase following SE-induced inflammation. Overproduction of reactive oxygen species or reactive nitrogen species leads to mitochondrial dysfunctions, DNA damage, and neuronal death[16]. In this study, a significantly higher level of TAC was found in *O. sanctum*-treated rats (either alone or in combination with VPA) as compared to the SE control, demonstrating its antioxidant potential. This is consistent with our previous studies where *Ocimum per se* or in combination with ASMs such as carbamazepine, phenytoin, levetiracetam, or VPA potentially reduced epilepsy-induced malondialdehyde, and increased reduced glutathione, and superoxide dismutase level in rats[6–8]. A recent study has also demonstrated the antioxidant potential of *O. sanctum* in animal models of Alzheimer's disease[15] and even morin, a flavonoids constituent of *Ocimum* has shown antioxidant effect in pentylenetetrazol-induced seizure model[17].

NSE is a relevant and reliable biomarker for the assessment of neuronal injury and tissue damage following brain insult and may serve as a predictive marker for evaluating the severity of neuronal damage associated with epilepsy[18]. Studies reported elevated levels of NSE following SE and TLE patients[19]. A recent study suggested the potential of NSE as a biomarker of electroencephalogram activity and for the assessment of the risk of seizure recurrence[20]. A previous study reported a significant reduction of NSE levels following VPA treatment compared to disease control rats[21]. In this study, NSE was significantly higher in the SE control compared to other drug-treated groups, suggesting extensive neuronal injury following SE. NSE was comparatively lower in the combination group compared to VPA or OSHE treatment alone, indicating that *O. sanctum* may have the potential to attenuate the neuronal damage and potential complications following SE.

As evident in human and animal studies, SE causes significant cerebral damage, increases the risk of developing subsequent epileptic episodes, and is associated with a distinct pattern of neuronal death in the hippocampus, such as in the hilus of dentate gyrus, CA3, and CA1 subfield[14]. In our study, extensive myelin sheath and axonal damage were found in the hippocampus of the SE control group compared to other drug-treated groups suggesting SE-induced neuronal death. The damage was alleviated in all treatment groups. Additionally, these results are further confirmed in the IHC assessment, where we found reduced neuronal loss and reactive astrocytes after *O. sanctum* treatment alone or in combination with VPA. According to the previous literature, VPA has shown a neuroprotective effect in neurodegenerative conditions by inhibiting histone-deacetylases and delaying apoptosis in degenerating neurons[22]. In another study, treatment with rosmarinic acid, a constituent of *O. sanctum* exerted neuroprotection by preventing the neuronal damage in the CA1 and CA3 regions of the hippocampus caused by kainite-induced seizures[23]. According to these findings, *O. sanctum* may have neuroprotective potential by preserving the neuronal echotexture and attenuating the SE-induced neuronal loss in the hippocampus.

This study has several limitations. The effect of chronic administration of *O. sanctum* (long-term) on cognition and neuronal injury post-SE has not been assessed in this study as we have only administered the drug for a shorter duration. We have used the lithium-pilocarpine model of SE to evaluate the adjuvant role of *O. sanctum* as it closely mimics the clinical characteristics of TLE in humans. However, for better translational value, other experimental models of SE should also be used alongside this, as no single test can demonstrate the anticonvulsant properties of new compounds satisfactorily. For example, phenobarbitone, tiagabine, and gabapentin block seizures in a pentylenetetrazol model which is an optimum model for screening for efficacy of drugs against absence seizures; but are ineffective against absence seizures in humans[24]. In this study, the assessment of memory and learning in rats was based on only two neurobehavioural tests (PA and EPM). This may be expanded to a battery of tests for a better understanding of its efficacy on cognition. In addition, the sequelae of SE like neurodegeneration are mainly associated with excitotoxicity, so the protective effect of *O. sanctum* on glutamate and other excitatory neurotransmitter-mediated pathway needs to be studied.

In conclusion, our study revealed that *O. sanctum* either alone or in combination with VPA led to better seizure control in the lithium-pilocarpine-induced SE model in rats. It reduced the SE-induced oxidative stress, improved memory, and attenuated neuronal loss and degeneration when administered in combination with VPA in the SE model. These findings suggest that *O. sanctum* may be an anticonvulsant and neuroprotective agent. Hence, further studies investigating its individual ingredients' effect and adjuvant potential in patients with epilepsy are needed, which will have translational value in epilepsy research. Moreover, a long-term study is required to investigate its chronic effect on SE and its sequelae in various experimental animal models of SE.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Funding

The authors received no extramural funding for the study.

Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

SCS designed the study and approved final version to be published. SCS, SSP, DS, and SS conducted the study, performed the analytic calculations and drafted the article. SCS, MT and SSP contributed to critical revision of the article. TCN facilitated transmission electron microscopy assessment and image interpretation. AN facilitated the immunohistochemistry assessment and image interpretation.

References

[1] John R, Grahf D, Lochner A, Taylor R, Krupp S, Vohra T. Evaluation and treatment of adult status epilepticus in the emergency department. *Curr Emerg Hosp Med Rep* 2022; **10**(4): 107-115.

[2] Moradi F, Eslami F, Rahimi N, Koohfar A, Shayan M, Maadan, M, et al. Modafinil exerts anticonvulsive effects against lithium-pilocarpine-induced status epilepticus in rats: A role for tumor necrosis factor-α and nitric oxide signaling. *Epilepsy Behav* 2022; **130**: 108649.

[3] Hawkes MA, Hocker SE. Systemic complications following status epilepticus. *Curr Neurol Neurosci Rep* 2018; **18**(2): 1-9.

[4] Zenki KC, Kalinine E, Zimmer ER, Dos Santos TG, Mussulini BHM, Portela LVC, et al. Memantine decreases neuronal degeneration in young rats submitted to LiCl-pilocarpine-induced status epilepticus. *Neurotoxicology* 2018; **66**: 45-52.

[5] Reddy DS, Kuruba R. Experimental models of status epilepticus and neuronal injury for evaluation of therapeutic interventions. *Int J Mol Sci* 2013; **14**(9): 18284-18318.

[6] Sarangi SC, Pattnaik SS, Joshi D, Chandra PP, Kaleekal T. Adjuvant role of *Ocimum sanctum* hydroalcoholic extract with carbamazepine and phenytoin in an experimental model of acute seizures. *Saudi Pharm J* 2020; **28**(11): 1440-1450.

[7] Sarangi SC, Pattnaik SS, Katyal J, Kaleekal T, Dinda AK. An interaction study of *Ocimum sanctum* L. and levetiracetam in pentylenetetrazole kindling model of epilepsy. *J Ethnopharmacol* 2020; **249**: 112389.

[8] Sarangi SC, Joshi D, Kumar R, Kaleekal T, Gupta YK. Pharmacokinetic and pharmacodynamic interaction of hydroalcoholic extract of *Ocimum sanctum* with valproate. *Epilepsy Behav* 2017; **75**: 203-209.

[9] Modebadze T, Morgan NH, Pérès IA, Hadid RD, Amada N, Hill C, et al. A low mortality, high morbidity reduced intensity status epilepticus (RISE) model of epilepsy and epileptogenesis in the rat. *PLoS One* 2016; **11**(2): e0147265.

[10] Kim D, Kim JM, Cho YW, Yang KI, Kim DW, Lee ST, et al. Drug committee of korean epilepsy society. antiepileptic drug therapy for status epilepticus. *J Clin Neurol* 2021; **17**(1): 11-19.

[11] Falco-Walter JJ, Bleck T. Treatment of established status epilepticus. *J Clin Med* 2016; **5**(5): 49.

[12] Yasiry Z, Shorvon S. The relative effectiveness of five antiepileptic drugs in the treatment of benzodiazepine-resistant convulsive status epilepticus: A meta-analysis of published studies. *Seizure* 2014; **23**(3): 167-174.

[13] Romoli M, Mazzocchetti P, D'Alonzo R, Siliquini S, Rinaldi VE, Verrotti A, et al. Valproic acid and epilepsy: From molecular mechanisms to clinical evidence. *Curr Neuropharmacol* 2019; **17**(10): 926-946.

[14] Liu KM, Huang Y, Wan PP, Lu YH, Zhou N, Li JJ, et al. Ursolic acid protects neurons in temporal lobe epilepsy and cognitive impairment by repressing inflammation and oxidation. *Front Pharmacol* 2022; **13**: 877898.

[15] Nandini HS, Krishna KL, Apattira C. Combination of *Ocimum sanctum* extract and Levetiracetam ameliorates cognitive dysfunction and hippocampal architecture in a rat model of Alzheimer's disease. *J Chem Neuroanat* 2022; **120**: 102069.

[16] Lin TK, Chen SD, Lin KJ, Chuang YC. Seizure-inudced oxidative stress in status epilepticus: Is antioxidant beneficial? *Antioxidants (Basel)* 2020; **9**(11): 1029.

[17] Kandhare AD, Mukherjee AA, Bodhankar SL. Anti-epileptic effect of morin against experimental pentylenetetrazol-induced seizures *via* modulating brain monoamines and oxidative stress. *Asian Pac J Trop Biomed* 2018; **8**(7): 352-359.

[18] Shaik AJ, Reddy K, Mohammed N, Tandra SR, Rukmini MK, Baba KS. Neuron-specific enolase as a marker of seizure-related neuronal injury. *Neurochem Int* 2019; **131**: 104509.

[19] Beretta S, Coppo A, Bianchi E, Zanchi C, Carone D, Stabile A, et al. Neurologic outcome of postanoxic refractory status epilepticus after aggressive treatment. *Neurology* 2018; **91**(23): e2153-e2162.

[20] Hanin A, Demeret S, Denis JA, Nguyen-Michel VH, Rohaut B, Marois C, et al. Serum neuron-specific enolase: A new tool for seizure risk monitoring after status epilepticus. *Eur J Neurol* 2022; **29**(3): 883-889.

[21] Saha L, Bhandari S, Bhatia A, Banerjee D, Chakrabarti A. Anti-kindling effect of bezafibrate, a peroxisome proliferator-activated receptors alpha agonist, in pentylenetetrazole induced kindling seizure model. *J Epilepsy Res* 2014; **4**(2): 45-54.

[22] Biermann J, Grieshaber P, Goebel U, Martin G, Thanos S, Di Giovanni S, et al. Valproic acid-mediated neuroprotection and regeneration in injured retinal ganglion cells. *Invest Ophthalmol Vis Sci* 2010; **51**(1): 526-534.

[23] Khamse S, Sadr SS, Roghani M, Hasanzadeh G, Mohammadian M, Rosmarinic acid exerts a neuroprotective effect in the kainate rat model of temporal lobe epilepsy: Underlying mechanisms. *Pharm Biol* 2015; **53**(12): 1818-1825.

[24] Wahab A. Difficulties in treatment and management of epilepsy and challenges in new drug development. *Pharmaceuticals (Basel)* 2010; **3**(7): 2090-2110.

Publisher's note