RESEARCH PAPER

Intranasal Delivery Of Fosphentoin Loaded With Nano-Lipid Carriers (NLC) To Assess Behavioral Antiepileptic Activity Using Zebra Fish Model.

Dr. Arunkumar R[1*], Tamil Selvan M[2]

[1] Professor and Head, Department of pharmacology, Chettinad academy of research and education, Tamil Nadu, Kelambakkam, Chennai, E-mail Id- arunrmbbs1978@gmail.com,
Orcid ID- 0000-0002-7657-7530

[2]Research scholar, Department of pharmacology, Chettinad academy of research and education, Tamil Nadu, Kelambakkam, Chennai, E-mail Id- mrtamilselvan96@gmail.com,
Orcid ID- 0009-0007-0051-7287

***Corresponding author:** Dr. Arunkumar R
Mail Id- arunrmbbs1978@gmail.com, Orcid ID- 0000-0002-7657-7530

Abstract:
Background: Epilepsy is a chronic neurological disorder characterized by recurrent seizures, and effective drug delivery remains a key challenge in achieving optimal therapeutic outcomes. Nanostructured lipid carriers (NLCs) have emerged as a promising system to enhance the bioavailability and brain delivery of antiepileptic drugs.
Objective: The present study aimed to evaluate the anticonvulsant efficacy of NLC-loaded Fosphenytoin in comparison with the standard fosphenytoin formulation using a pentylenetetrazole (PTZ)-induced seizure model in experimental animals.
Methods: Animals were divided into control, standard drug (fosphenytoin), and test drug (NLC-fosphenytoin) groups. Seizures were induced using PTZ, and parameters such as onset of convulsions, duration of seizures, and percentage protection were recorded. The anticonvulsant activity of the test formulation was assessed and compared with the standard treatment.
Results: The NLC-fosphenytoin group showed a significant delay in the onset of seizures and a reduction in seizure severity and duration when compared to the control group. The test formulation demonstrated comparable or improved anticonvulsant efficacy relative to the standard drug.
Conclusion: The findings suggest that NLC-based delivery of fosphenytoin enhances anticonvulsant activity in the PTZ model and may offer a promising strategy for improved management of epilepsy.

Keywords- NLC, Antiepileptics, PTZ, Fosphenytoin, NLC+Fosphetoin

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Introduction

Epilepsy is a chronic neurological disorder characterized by recurrent seizures and affects millions of people worldwide. Rapid and effective suppression of seizures remains a major clinical challenge, particularly in acute settings where delayed drug action and limited brain penetration can compromise therapeutic outcomes [1]. Conventional antiepileptic drug administration routes, including oral and parenteral delivery, are often associated with delayed onset of action, systemic adverse effects, and the need for trained medical personnel in emergency situations [2]. These limitations have driven the search for alternative drug delivery strategies capable of achieving rapid and targeted delivery of antiepileptic agents to the brain.

Intranasal drug delivery has emerged as a promising non-invasive approach for direct nose-to-brain transport, bypassing the blood–brain barrier through olfactory and trigeminal nerve pathways. This route offers advantages such as rapid onset of action, reduced systemic exposure, and improved patient convenience, making it particularly attractive for the management of acute neurological conditions including epilepsy [3,4].

Fosphenytoin, a phosphate ester prodrug of phenytoin, is widely used in the management of acute seizures and status epilepticus due to its improved aqueous solubility and reduced local tissue toxicity compared to phenytoin [5]. Despite these advantages, fosphenytoin is conventionally administered via intravenous or intramuscular routes, which may limit its use in pre-hospital or resource-limited settings. Exploring alternative non-invasive delivery strategies for fosphenytoin, particularly for rapid brain targeting, may offer clinically meaningful benefits in emergency seizure management.

Nanostructured lipid carriers (NLCs) have gained attention as advanced drug delivery systems capable of

*Author for Correspondence: arunrmbbs1978@gmail.com

enhancing drug solubility, stability, and permeation across biological membranes. NLC-based formulations have shown promise in improving intranasal drug transport to the brain by prolonging nasal residence time, facilitating mucosal permeation, and enhancing neuronal uptake [6]. Incorporation of antiepileptic drugs into lipid-based nanocarriers may therefore improve nose-to-brain delivery efficiency and therapeutic performance compared to conventional formulations.

Zebrafish (Danio rerio) have emerged as a robust and translational in vivo model for neurological disorders, including epilepsy, owing to their genetic homology with humans, conserved neurochemical pathways, optical transparency, and suitability for high-throughput pharmacological screening [7]. Chemically induced seizure models using pentylenetetrazole (PTZ) are well established in zebrafish and exhibit reproducible seizure-like behaviors that correlate with neuronal hyperexcitability [8].

Behavioral endpoints in zebrafish, including seizure severity scoring, abnormal swimming patterns, hyperlocomotion, clonus-like convulsions, and recovery time, provide sensitive functional measures of central nervous system drug activity. Quantitative locomotor tracking further allows objective assessment of seizure suppression and neurobehavioral recovery following pharmacological intervention [9].

In the present study, PTZ-induced seizures in zebrafish were employed to evaluate the anticonvulsant efficacy of an intranasally administered nanostructured lipid carrier-based fosphenytoin formulation (NLC + fosphenytoin) in comparison with intranasal fosphenytoin solution as the standard formulation. Behavioral parameters including seizure severity, locomotor activity, and recovery time were used to assess the therapeutic performance and onset of action of the test formulation. This study aims to provide in vivo behavioral evidence supporting the potential of intranasal NLC-based fosphenytoin delivery as an effective nose-to-brain strategy for rapid seizure control.

Methodology

Intranasal Administration Protocol (Nose-to-Brain Delivery)

Route of Administration:

Both the standard drug (fosphenytoin solution) and the test formulation (NLC + fosphenytoin) were administered via the intranasal (IN) route to facilitate direct nose-to-brain transport.

Preparation of Animals:

Adult zebrafish were fasted for 6 h prior to dosing and acclimatized to the procedure room for 30 min. Fish were gently anesthetized using tricaine methanesulfonate (MS-222; 80–100 mg/L) until loss of righting reflex. Each fish was then placed dorsally on a moist sponge under a stereomicroscope to expose the nares while maintaining gill perfusion with aerated system water.

Dosing Volume and Technique:

A calibrated micro-pipette fitted with a fine tip was used to instill the formulation. A total volume of 2 µL per fish (1 µL per nostril) was administered dropwise onto the external nares, allowing capillary action to draw the formulation into the nasal cavity. Care was taken to avoid oral or branchial exposure and to prevent overflow. After administration, fish were maintained on the moist support for 30–60 s to allow nasal absorption and then transferred to recovery tanks with fresh system water.

Formulation Considerations:

Formulations were freshly prepared, isotonic, and maintained at room temperature. The NLC formulation was gently vortexed prior to dosing to ensure homogeneity. No permeation enhancers were used to avoid mucosal irritation.

Post-dosing Recovery and Monitoring:

Fish were observed continuously until full recovery of spontaneous swimming (typically within 2–3 min). Animals showing signs of respiratory distress or dosing-related regurgitation were excluded. No mortality or overt nasal irritation was observed.

Timing Relative to PTZ:

Intranasal dosing of fosphenytoin (standard) or NLC + fosphenytoin (test) was performed 30 min prior to PTZ exposure. Behavioral testing was conducted at 30 min, 45 min, and 1 h post-treatment.

Literature Basis:

Intranasal delivery in adult zebrafish using microliter volumes per nostril has been reported to achieve rapid CNS exposure and is suitable for evaluating nose-to-brain drug transport. This protocol is adapted from established intranasal dosing methodologies in zebrafish and nose-to-brain delivery literature. (7–9)

In vivo adult zebrafish behavioural test

Dose Selection and Treatment Protocol

PTZ-Induced Seizure Model (Adult Zebrafish):

Seizures were induced in adult zebrafish using pentylenetetrazole (PTZ) by immersion at a concentration of 6 mM for 10 minutes. This dose reliably produces stage-wise seizure-like behavior, hyperlocomotion, clonus-like convulsions, and post-ictal behavioral suppression in adult zebrafish and is widely used for anticonvulsant screening (1,2).

Standard Drug: Fosphenytoin (Intranasal Solution):

Fosphenytoin sodium (powder form) obtained from Trifarma (Italy) was reconstituted in sterile normal saline. As intranasal fosphenytoin dosing in zebrafish is not standardized, the dose was selected based on published anticonvulsant validation studies using phenytoin in adult zebrafish (80 mg/kg, i.p.) and

converted to a phenytoin-equivalent dose. Accordingly, fosphenytoin was administered intranasally at a phenytoin-equivalent dose of 20 mg/kg (3,4).

Test Drug: NLC + Fosphenytoin (Intranasal Delivery):
The test formulation consisted of fosphenytoin loaded into nanostructured lipid carriers (NLCs) and administered intranasally at the same phenytoin-equivalent dose of 20 mg/kg to enable direct comparison with the standard formulation. Lipid-based nanocarriers enhance nose-to-brain delivery and improve brain targeting of antiepileptic drugs (5,6).

Treatment Schedule:
Adult zebrafish were pre-treated with fosphenytoin (standard) or NLC + fosphenytoin (test) 30 minutes prior to PTZ exposure. Behavioral assessments were performed at 30 minutes, 45 minutes, and 1 hour post-treatment.
Behavioral testing was carried out from 9:00 AM to 5:00 PM in a designated testing room with standardized environmental conditions. Adult zebrafish (n = 6 per group) were maintained in their home tanks and allowed to acclimate for at least 15 days before experiments began. Animals were randomly assigned to treatment groups and kept in separate tanks to limit variability arising from environment or handling. All behavioural testing and analyses were conducted by a blinded investigator to prevent bias.

Novel tank test
Anxiety-like behaviour was evaluated using the novel tank diving test (n = 6/group). Adult zebrafish were tested individually in clear acrylic tank (36 × 8 × 12 cm) containing distilled water, maintained at 27 ± 1 °C under standardized lighting. Each fish was carefully placed into the tank and its behaviour was video-recorded for 2 min with a Logitech C500 camera using both side and top views. The arena was digitally partitioned into upper and lower regions, and measures included time spent in each region, latency to first enter the upper region, freezing time, and the number of erratic swimming bouts. Between trials, tanks were washed and refilled to eliminate residual cues. Video scoring was conducted using *fish_tracker.ipyn*.

Locomotor activity test
Locomotor performance was evaluated using the same experimental context to minimize contextual effects. Each fish was tested individually in a 5 L glass tank and recorded for 2 min. Total distance moved, average swimming speed, and the proportion of time spent across predefined speed categories were quantified using fish_tracker.ipynb. These outcomes were used to estimate baseline motor activity and habituation to the unfamiliar arena.\

Plus-maze test
Adult zebrafish (n = 6 per group) were pre-exposed to the testing conditions for 30 min at 27 ± 1 °C before the assay. Learning and memory were evaluated in a plus-maze consisting of four differently colored arms (blue, green, red, and black). During a 5-day training period, one arm was consistently associated with a food reward. Each fish was permitted to explore the maze for 2 min daily. On the test day, the reward was omitted and behaviour was captured using an overhead camera. The duration spent in, and entries made into, each arm was quantified to determine color-linked associative learning and memory retention.

Results
Locomotor activity test

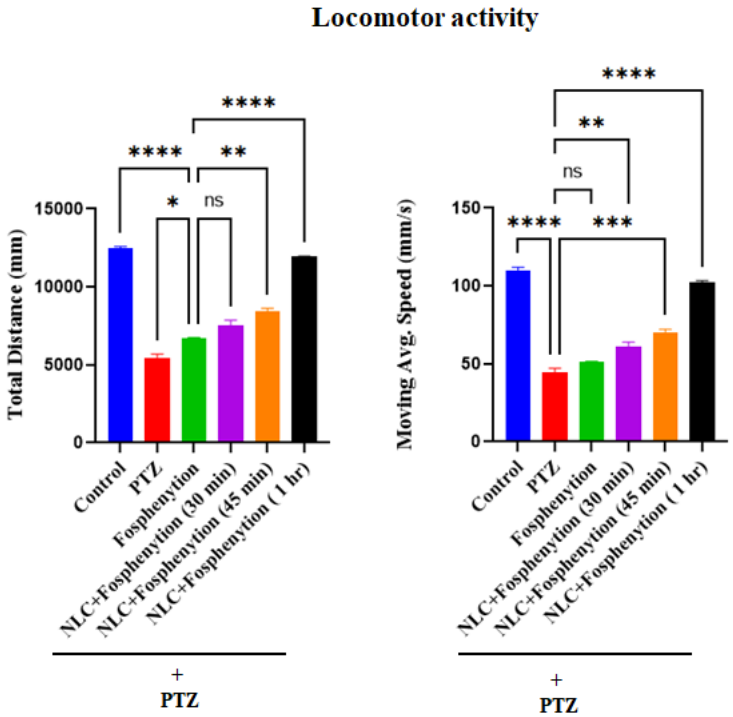

Figure ( ). Effect of NLC-Fosphenytoin on PTZ-induced locomotor deficits in adult zebrafish: (A) Total distance travelled (mm). (B) Mean swimming speed (mm/s). Adult zebrafish (n = 6/group) were treated with PTZ followed by NLC-Fosphenytoin and assessed at 30 min, 45 min, and 1 h post-treatment. Data are expressed as mean ± SEM. Statistical significance is indicated as ns (not significant), **p < 0.01, ***p < 0.001, and ****p < 0.0001.

PTZ exposure significantly impaired locomotor performance in adult zebrafish. As shown in Figure ( ), total distance travelled (A) and mean swimming speed (B) were markedly reduced in the PTZ group compared to controls (****p < 0.0001). Control fish exhibited normal exploratory behaviour, whereas PTZ-treated fish demonstrated nearly a 50% decline in both parameters, indicating pronounced locomotor suppression. Treatment with NLC-Fosphenytoin produced a progressive and time-dependent recovery. At 30 min post-treatment, modest improvement was observed compared to the PTZ group, with some comparisons remaining non-significant (ns). At 45 min,

both total distance and average speed were significantly increased relative to PTZ (**p < 0.01 to ***p < 0.001). At 1 h post-treatment, locomotor parameters were significantly restored (****p < 0.0001 vs. PTZ) and approached control levels. These results demonstrate that PTZ induces significant motor deficits, while NLC-Fosphenytoin effectively reverses these impairments over time.

Novel tank test

PTZ exposure significantly altered behavioural responses in the novel tank test. As shown in Figure ( ), PTZ-treated zebrafish exhibited a marked reduction in total distance travelled (A) and mean swimming speed (B) compared to controls (****p < 0.0001), indicating suppressed exploratory behaviour. In terms of anxiety-related parameters, PTZ significantly decreased the time spent in the upper zone (C) while increasing the time spent in the bottom zone (D) relative to controls (****p < 0.0001), reflecting enhanced anxiety-like behaviour. Administration of NLC-Fosphenytoin resulted in a time-dependent improvement across all behavioural endpoints. At 30 min post-treatment, partial recovery was observed, with modest increases in locomotor activity and upper zone exploration, though some comparisons remained non-significant (ns). At 45 min, significant improvements were noted in total distance, swimming speed, and time spent in the upper zone (**p < 0.01 to ***p < 0.001 vs. PTZ). By 1 h post-treatment, behavioural parameters were markedly restored (****p < 0.0001 vs. PTZ), approaching control levels, with increased upper zone exploration and reduced bottom-dwelling behaviour. These results evaluate that PTZ induces pronounced anxiety-like and locomotor deficits, while NLC-Fosphenytoin progressively ameliorates these behavioural impairments.

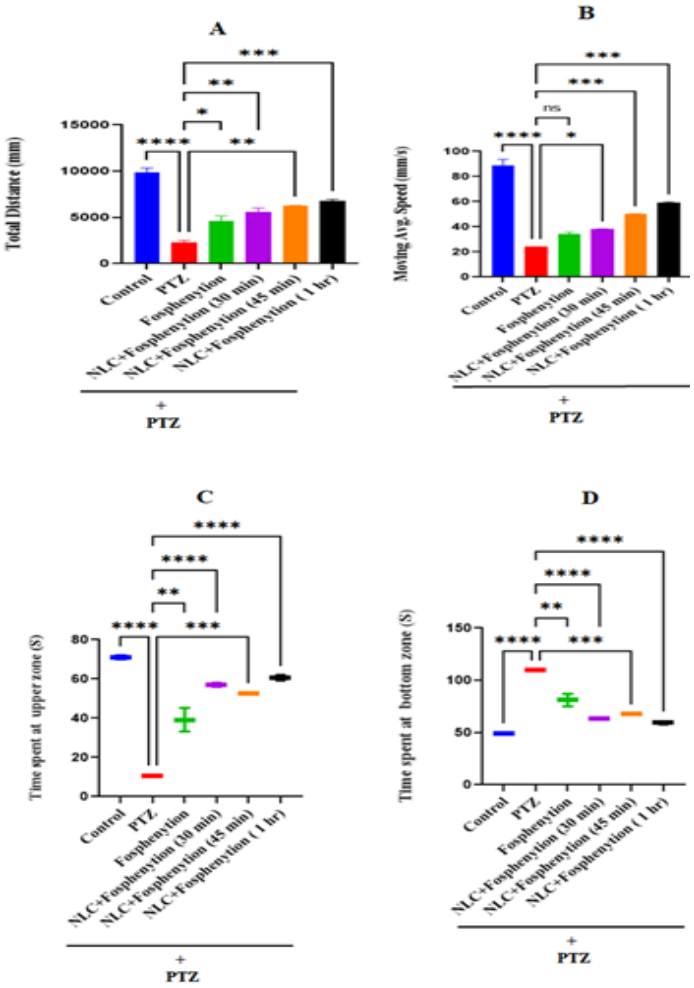

Figure ( ). Effect of NLC-Fosphenytoin on PTZ-induced behavioural alterations in the novel tank test. (A) Total distance travelled (mm). (B) Mean swimming speed (mm/s). (C) Time spent in the upper zone (s). (D) Time spent in the bottom zone (s). Adult zebrafish (n = 10/group) were exposed to PTZ followed by NLC-Fosphenytoin treatment and assessed at 30 min, 45 min, and 1 h post-treatment. Data are presented as mean ± SEM. Statistical significance is indicated as ns (not significant), *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Plus-maze test

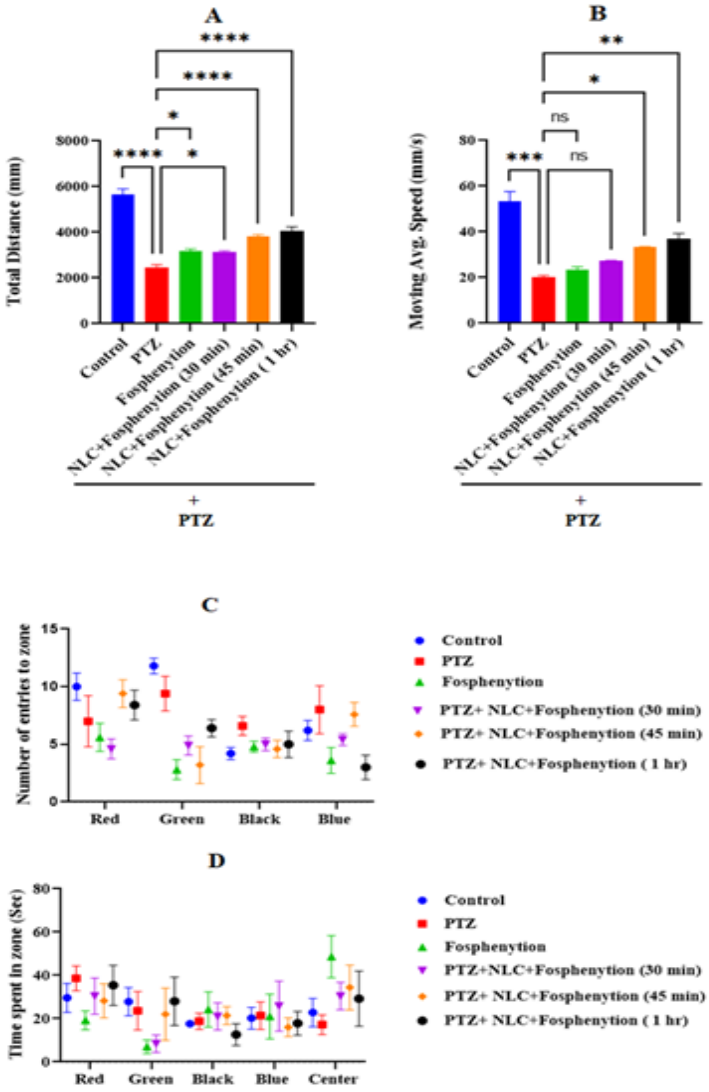

Figure ( ) Plus-maze performance and zone exploration in adult zebrafish. (A) Total distance travelled (mm) and (B) mean swimming speed (mm/s) recorded during the plus-maze trial. (C) Number of entries into each coloured arm (red, green, black, blue). (D) Time spent (s) in each arm. Data are presented as mean ± SEM. Statistical significance is indicated as ns (not significant), *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

PTZ administration significantly impaired locomotor performance in the plus-maze paradigm. As shown in Figure A, total distance travelled was markedly reduced in the PTZ group compared with control (****p < 0.0001). Mean swimming speed was also significantly decreased following PTZ exposure (***p < 0.001 vs control; Figure B), confirming suppression of exploratory behaviour. Fosphenytoin alone produced a moderate increase in total distance compared with PTZ (*p < 0.05), though improvement in swimming speed remained statistically non-significant (ns). In contrast, treatment with PTZ + NLC-Fosphenytoin demonstrated a time-dependent recovery. Total distance travelled increased progressively at 30 min and was significantly elevated at 45 min and 1 h compared to PTZ (****p < 0.0001). Mean swimming speed showed partial recovery at 30 min (ns), with significant improvement observed at 45 min (*p < 0.05) and further enhancement at 1 h (**p < 0.01 vs PTZ). Analysis of spatial exploration (Figure C and D) revealed that PTZ altered the distribution of arm entries and time spent across colored arms (red, green, black, and blue), indicating disrupted exploratory patterning. NLC-Fosphenytoin groups exhibited partial normalization of both the number of entries and time allocation across zones over time, particularly at 45 min and 1 h, suggesting restoration of organized exploratory behaviour. Overall, PTZ induced significant locomotor suppression and altered spatial exploration in the plus-maze test, whereas NLC-Fosphenytoin produced a progressive and significant recovery of behavioural performance.

Discussion.

The present study investigated the anticonvulsant efficacy of intranasally administered fosphenytoin-loaded nanostructured lipid carriers (NLC + fosphenytoin) in a PTZ-induced seizure model in adult zebrafish, with intranasal fosphenytoin solution used as the standard treatment. PTZ exposure produced pronounced behavioral impairments, including reduced locomotor activity, altered exploratory behavior in the novel tank test, and disrupted spatial exploration in the plus-maze task, consistent with chemically induced seizure phenotypes reported in adult zebrafish models. These findings confirm the suitability of the PTZ model for evaluating antiseizure interventions in zebrafish.

Intranasal administration of the standard drug, fosphenytoin (procured from Trifarma), produced partial improvement in PTZ-induced behavioral deficits, indicating that fosphenytoin retains anticonvulsant activity when delivered via the nasal route. However, the recovery observed with intranasal fosphenytoin solution was modest and slower compared with the NLC-based formulation. This difference may be attributed to limited nasal residence time and suboptimal mucosal permeation of the aqueous fosphenytoin solution, which can restrict the extent and rate of drug transport to the brain.

In contrast, treatment with NLC + fosphenytoin resulted in a marked and time-dependent restoration of locomotor activity, exploratory behavior, and spatial performance, with significant improvements evident at 45 min and near-normalization of behavior by 1 h post-treatment. The superior efficacy of the NLC formulation can be attributed to the known advantages of lipid-based nanocarriers for intranasal nose-to-brain delivery, including prolonged nasal residence, enhanced permeation across the nasal epithelium, and facilitation of direct transport along olfactory and trigeminal pathways. These mechanisms likely contributed to faster and higher brain exposure of fosphenytoin when delivered in the NLC system compared with the free drug solution.

Behavioral recovery in the novel tank test and plus-maze paradigm further supports the neurofunctional benefits of the NLC formulation. PTZ-induced anxiety-like behavior, reflected by increased bottom-dwelling and reduced upper-zone exploration in the novel tank test, was progressively reversed by NLC + fosphenytoin. Similarly, disrupted exploration patterns in the plus-maze were partially normalized following NLC-based treatment. These observations suggest that the NLC formulation not only suppresses seizure-related motor deficits but also ameliorates PTZ-associated alterations in anxiety-like and exploratory behaviors, indicating broader functional recovery of central nervous system activity.

The intranasal route used in this study offers translational relevance, as it provides a non-invasive strategy for rapid brain targeting of antiepileptic agents while minimizing systemic exposure. Fosphenytoin is conventionally administered via parenteral routes in acute clinical settings; therefore, successful demonstration of intranasal delivery in an in vivo zebrafish model highlights the potential of this approach for developing patient-friendly alternatives for emergency seizure management. Although direct pharmacokinetic measurements were not performed, the faster onset and greater behavioral recovery observed with NLC + fosphenytoin indirectly support enhanced brain delivery via the nose-to-brain pathway.

Certain limitations of the present study should be acknowledged. The sample size was limited (n = 6 per group) in accordance with IEC approval, and the study was designed as a pilot investigation. Future studies with larger sample sizes, quantitative seizure scoring, and direct measurement of brain drug concentrations will be valuable to further validate the pharmacokinetic and pharmacodynamic advantages of the NLC formulation. Additionally, histopathological evaluation of nasal mucosa and brain tissue would help confirm the safety of repeated intranasal administration of lipid-based nanocarriers.

Overall, the findings of this study demonstrate that intranasal delivery of fosphenytoin using nanostructured lipid carriers significantly enhances anticonvulsant efficacy in a PTZ-induced adult zebrafish model compared with conventional intranasal fosphenytoin solution. This work provides experimental support for the potential of NLC-based nose-to-brain delivery systems as a promising strategy for rapid and non-invasive seizure management and warrants further translational investigation in higher animal models.

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