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Evaluation of sodium valproate loaded nanoparticles in acute and chronic pentylenetetrazole induced seizure models

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ABSTRACT

Background and purpose: Efficacy of sodium valproate in epilepsy is limited by its poor blood brain barrier penetration and side effects. Nanoparticles may offer a better drug delivery system to overcome these limitations. This study evaluated the efficacy of sodium valproate encapsulated in nanoparticles in pentylenetetrazole (PTZ) induced acute and kindling models of seizures in male Wistar rats.

Methods: Poly lactic-co-glycolic acid (PLGA) based, polysorbate 80 stabilized sodium valproate loaded nanoparticles (nano sodium valproate) and rhodamine loaded nanoparticles (RLN) were formulated by double emulsion- solvent evaporation method and characterized for their size, shape, zeta potential and drug loading percentage. RLN was used to demonstrate blood brain barrier (BBB) permeability of nanoparticles. Serum drug levels were estimated using high performance liquid chromatography. The efficacy of standard sodium valproate (300 mg/kg) and nano sodium valproate (~300, ~150 and ~75 mg/kg of sodium valproate) were evaluated in experimental animal models of seizures along with their effects on behavioral and oxidative stress parameters. Drugs were administered 60 min before PTZ in acute model. In the kindling model, drugs were administered every day while PTZ was administered on alternate days 60 min after drug administration. All the study drugs/compounds were administered intraperitoneally.

Results: RLN were observed to be clustered in cortex which implied that the nanoparticles crossed BBB. Both standard sodium valproate and nano sodium valproate reached therapeutic serum level at 15 min and 1 h, but were undetectable in serum at 24 h. In acute PTZ (60 mg/kg) model, nano sodium valproate (~300 mg/kg of sodium valproate) and standard sodium valproate showed protection against seizures till 6 h and 4 h, respectively. There were significant behavioral impairment and oxidative stress with standard sodium valproate in acute model as compared to nano sodium valproate at 6 h. In kindling model, induced with PTZ (30 mg/kg, every alternate day for 42 days), complete protection from seizures was observed with nano sodium valproate (~150 mg/kg and ~75 mg/kg of sodium valproate) and standard sodium valproate (300 mg/kg). Similarly, significant protection from behavioral impairment and oxidative stress was observed with standard sodium valproate and nano sodium valproate as compared to PTZ.

Conclusion: When compared to conventional therapy, nano sodium valproate showed protection from seizures at reduced doses and for a longer duration in animal models of epilepsy. This study suggests the potential of nano sodium valproate in the treatment of epilepsy.

1. Introduction

Epilepsy is a chronic neurological disorder manifesting as frequent unprovoked seizures and affects approximately 1% of population (Mehndiratta and Wadhai, 2015). An Indian study estimated that approximately ten million people suffer from epilepsy, three million from

Abbreviations: PTZ, Pentylenetetrazole; BBB, blood brain barrier; CSF, cerebrospinal fluid; CNS, central nervous system; RES, reticuloendothelial system; PLGA, poly lactic-co-glycolic acid; IAEC, Institutional Animal Ethics Committee; kD, kiloDalton; TEM, transmission electron microscope; HPLC, high performance liquid chromatography; i.p., intraperitoneal; GTCS, generalized tonic clonic seizures; MDA, malondialdehyde; GSH, glutathione; ANOVA, one way analysis of variance; LDL, low density lipoprotein

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drug resistant epilepsy and one million are likely to undergo epilepsy surgeries (Rao, 2017). Blood brain barrier (BBB) dysfunction is a known etiology for both seizure disorder and drug resistant epilepsy. Currently available antiepileptic drugs also have frequent distressing adverse effects (Jahromi et al., 2011; Mattson et al., 1985; Perucca, 2002). Thus, although epilepsy is a common disorder; antiepileptic drugs do not show the optimum efficacy in terms of clinical expectation, patient compliance and seizure free interval.

Sodium valproate a broad-spectrum antiepileptic drug, is used in absence seizure, generalized tonic-clonic seizure, myoclonic seizure, secondarily generalized tonic-clonic seizure, Lennox-Gastaut syndrome and bipolar disorder (Emrich et al., 1985; Ferrie and Patel, 2009; Mattson et al., 1992; Perucca, 2002; Ramakrishnappa and Belhekar, 2013). There appears to be poor correlation between plasma concentration and efficacy due to asymmetric BBB transport (Stapleton et al., 2008). Moreover, sodium valproate has many side effects such as transient gastrointestinal symptoms, central nervous system (CNS) effects which include sedation, tremor, ataxia; rash, alopecia, appetite stimulation, weight gain, elevation of hepatic transaminases in plasma, fulminant hepatitis, acute pancreatitis, hyperammonemia, teratogenic effects such as neural tube defect (Belcastro et al., 2013; Dreifuss et al., 1989; Ramakrishnappa and Belhekar, 2013; Wadzinski et al., 2007; Yaman et al., 2013).

Behavioral and cognitive impairments are known to occur in seizures (Berg, 2011; Mehla et al., 2010) and even after a single episode of seizure (Aldenkamp and Bodde, 2005). Moreover, antiepileptic drug itself leads to behavioral impairment (Kälviäinen et al., 1996; Reeta et al., 2009). Studies in both animals and humans have reported cognitive impairment with seizures and antiepileptic drugs (Gupta et al., 2003; Hessen et al., 2007; Kumar et al., 2018; Mehla et al., 2010; Mertens et al., 2018; Reeta et al., 2009, 2010). Therefore, in the present study we evaluated the effect of seizures, sodium valproate and its nanoformulation on cognitive and behavioral parameters in rats.

Nanotechnology can engineer strategies to deliver antiepileptic drugs to the brain such as encapsulating the drug within a nanoscale delivery system like polymeric nanoparticle, liposome, micelle, dendrimer, nanostructured lipid carrier etc. Polymeric nanoparticles possess physical and chemical properties, which make them suitable for surface modification for targeting specific tissues. They possess good bio-distribution, biodegradability and biocompatibility considered more pertinent for the targeted delivery of wide range of therapeutic agents (Faraji and Wipf, 2009).

Once nanoparticles enter into the blood, they appear as foreign particles to reticuloendothelial system (RES) of body. They become opsonized, phagocytosed and are removed from blood circulation prior to reaching target site. These obstacles have been partly surmounted with poly lactic-co-glycolic acid (PLGA) nanoformulation. Moreover, PLGA offers good sustained release property and due to surface functional groups, it provides surface decorating possibilities on nanoparticles (Faraji and Wipf, 2009). Stabilizing agents like polysorbate 80 contribute to sterically stabilize nanoparticles, escape RES, impart prolonged circulation life, and low immunogenicity (Benvegnú et al., 2012; Bender et al., 2012; Chacko et al., 2018). Polymeric matrices prevent degradation of drug and provide its sustained release from these nanoparticles (Faraji and Wipf, 2009). Polysorbate 80 also helps in increasing permeation through BBB (Georgieva et al., 2014; Kreuter et al., 2002). It is suggested that these properties of nanoparticles would decrease dose, duration and consequent side effects of antiepileptic drugs such as cognitive and behavioral impairment along with protection from seizures and its complications. Pentylenetetrazole (PTZ) blocks $GABA_A$ receptor and is used to induce acute and chronic models of seizures. In this study, we have evaluated antiepileptic effect of nanoparticles in PTZ induced acute and chronic models of seizures, their effects on cognition and, oxidative stress.

2. Materials & methods

2.1. Materials

The following chemicals were utilized in the study: PLGA (with molecular weight ratio 65:35, Sigma Aldrich Ltd, Unites States), span 20 (Merck Ltd, India), polysorbate 80 (Merck Ltd, India), poloxamer 188 (Merck Ltd, India), sodium valproate (Sigma Aldrich Ltd, Unites States), pentylenetetrazole (Sigma Aldrich Ltd, Unites States), acetic acid (Merck Ltd, India), thiobarbituric acid, sodium dodecyl sulphate, n-butanol:pyridine (15:1), tetraethoxypropane, 5% trichloroacetic acid, 5′5- dithiobis (2-nitrobenzoic acid), reduced glutathione (Sigma Aldrich Ltd, Unites States). Dialysis tube with molecular weight cut off- 2 kDa (Sigma Aldrich, Unites States) was procured for dialysis bag technique. The chemicals used in HPLC were of HPLC grade; others were of analytical grade.

2.2. Animals

Male Wistar rats (weight 150–200 g) were used for the study. They were kept in polyacrylic cages with ≤ 4 animals per cage. All animals had access to food and water ad libitum. Acclimatization to the laboratory environment was done for all animals for 7 days before conducting experiments. Animals were divided into groups of 6 each. Ethical approval was obtained from Institutional Animal Ethics Committee (IAEC) before starting animal experiments (Ethics approval no-711/IAEC/2013). All experiments were done midday. All necessary precautions were taken to minimize suffering and to reduce the number of animals for experimentation. Utmost care was taken in handling and sacrificing the animals (as per ARRIVE guidelines and National Institutes of Health (NIH) guidance for laboratory animals).

2.3. Formulation of sodium valproate loaded polymeric nanoparticles

Sodium valproate loaded, polysorbate 80 coated, PLGA based polymeric nanoparticles (nano sodium valproate) were synthesized using double emulsion- solvent evaporation technique (Murakami et al., 1999; Peltonen et al., 2002). 50 mg of PLGA (molecular weight ratio 65:35) was dissolved in 5 ml of acetonitrile to form organic phase. 300 mg of sodium valproate was dissolved in deionized water and 60 mg of span 20 was added into it to form aqueous phase 1 under continuous magnetic stirring. It was mixed with organic phase to form water-in-oil emulsion (E1). 100 mg of polysorbate 80 was dissolved in deionized water to form aqueous phase 2. Emulsion formulated earlier (E1) was added into aqueous phase 2 and left overnight for solvent evaporation under continuous magnetic stirring. The nanoparticles were dialyzed using dialyzing membrane having pore size of 2 kDa followed by centrifugation at 12,000 rpm for 10 min. The pellet was mixed with mannitol as cryoprotectant and deionized water to form 1% mannitol solution and was lyophilized using Allied Frost Lyophilizer FD 5 (Varshosaz et al., 2012). The dried powder was weighed and stored at 4 °C.

2.4. Characterization of nano sodium valproate

2.4.1. Size, shape and zeta potential

Size of the nanoparticles was studied using NANOSIGHT (LM20, Malvern Instrument, United Kingdom). Transmission electron microscope (TEM) (Techni FEI G2) was used to study shape and integrity of the nanoparticles. Zeta potential was measured with Zetasizer^{TM} system (Malvern Instrument, United Kingdom).

2.4.2. Drug loading assay

Drug loading percentage was estimated with high performance liquid chromatography (HPLC) (Waters 1525 with binary pump and UV detector) using C-18 column (Waters Sunfire C-18 BDS column, Ireland)

at detection wavelength of 210 nm (British Pharmacopoeia, 2014). Mobile phase had a flow rate of 2 ml/min and constituted of potassium dihydrogen orthophosphate (0.32% w/v) with pH adjusted to 3.5 and, orthophosphoric acid and acetonitrile (in a ratio of 45:55). Drug loaded nanoparticles (100 mg) were dissolved in 1 ml of dichloromethane. Drug was extracted with 1 ml double distilled water. After centrifugation, 20 µl of sample was injected. Drug loading percentage and encapsulation efficiency were calculated from the standard graph.

2.4.3. Drug release kinetics

Dialysis bag technique was used to study drug release kinetics at 15 min, 30 min, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h, 48 h, 96 h and 144 h intervals (D'Souza, 2014). 100 mg of lyophilized nano sodium valproate were reconstituted in 2 ml of saline phosphate buffer (pH 7.4) in a dialysis tube (2.0 kDa molecular weight cut-off). The tube was kept under mild stirring condition against saline phosphate buffer at 37 °C. At predetermined intervals, 500 µl sample was withdrawn from the receiver compartment and equal volume was replaced every time. Samples collected were subjected to spectrophotometric analysis at 210 nm using UV–vis spectrophotometer (Spectra Max, United States).

2.5. Study of distribution of nanoparticles in different areas of brain

Rhodamine-B loaded nanoparticles were formulated using the same technique as sodium valproate loaded nanoparticles (Vergoni et al., 2009). Rhodamine-B loaded nanoparticles were injected in male Wistar rat via intraperitoneal (i.p.) route. At 6 h, animal was sacrificed and, brain was dissected and frozen immediately in liquid nitrogen. Sections of 5 µm thickness were cut using microtome. Nucleus was stained with 4′, 6-diamidino-2-phenylindole and studied under fluorescent microscope at 358 nm excitation and 461 nm emission wavelengths. Images were captured.

2.6. Estimation of serum level of sodium valproate

Blood samples were collected at 15 min, 1 h and 24 h following i.p. administration of sodium valproate (300 mg/kg) and nano sodium valproate (~300 mg/kg of sodium valproate). 500 µl of serum was taken in an Eppendorf tube. 1000 µl of acetonitrile was added. At 13,000 rpm and 4 °C temperature, the mixture was centrifuged for 10 min. Supernatant and pellet was separated and 0.22 µm syringe filter was used for filtration. Sodium valproate levels analyzed in the filtrate using HPLC system (Waters HPLC 1525 with binary pump and UV detector).

2.7. Behavioural parameters

2.7.1. Elevated plus maze test

Elevated plus maze consisted of two crossed arms- one open and other closed, in the form of a PLUS sign, and a square shaped center. This wooden platform was placed at a height of 50 cm above the ground. The test was conducted in two phases. In the first phase on day one, initial transfer latency was obtained. The test involved placing a rat at the edge of an open arm facing away from center and noting down the time taken by the rat to enter any of the closed arms with all four limbs. This was termed as transfer latency. The maximum time limit was 60 s. After 24 h, the 2nd phase was conducted to record retention transfer latency using the same protocol (Reeta et al., 2011). The same procedure was applied to all the groups of animals (n = 6 in each group) in all seizure models.

2.7.2. Passive avoidance test

Passive avoidance task was conducted using a passive avoidance apparatus (UGO Basile, Italy). In this apparatus, there were two cubicles, one was lit and the other one was dark. These were separated by a guillotine door. The base of the dark cubicle was made of steel grids wired to produce electric shock. The test was conducted in two phases. In the first phase on day one, initial transfer latency was obtained. The procedure included habituation of the rat in the lit cubicle for 60 s followed by opening of the guillotine door. The time taken by the rat to enter into dark cubicle was noted down as initial transfer latency. The moment the rat entered into dark cubicle, the guillotine door was shut and an electric foot shock (75 V, 0.2 mA, 50 Hz) was applied to the floor grids for 3 s. The rat was removed from the dark cubicle after 5 s. In second phase on day two, the procedure was repeated to obtain retention transfer latency without applying foot shock. The maximum limit for latency time was 600 s (Reeta et al., 2009; Tsuji et al., 2003). The same procedure was applied to all the groups of animals (n = 6 in each group) in all seizure models.

2.8. Seizure induction

2.8.1. PTZ induced seizures

PTZ was freshly prepared by dissolving in cold normal saline and administered in a dose of 60 mg/kg, i.p. at 6 h after drug administration. This dose of PTZ has been standardized as 100% convulsant dose with minimum death rate observed in rats in our laboratory. The latency to myoclonic jerks was noted along with occurrence of generalized tonic clonic seizures (GTCS). The loss of righting reflex was also monitored. The observation period was 30 min for individual rat (n = 6 in each group) (Malhotra and Gupta, 1997).

2.8.2. PTZ induced kindling

There were 6 animals per group and sub-convulsive dosage of PTZ (30 mg/kg) was administered intraperitoneally on every other day (48 ± 1 h). The rats were monitored for 30 min after each dose for convulsive behavior (Reeta et al., 2009). Seizure activity was evaluated using the Racine scale as reported by Racine, 1972:

Following parameters were recorded

i The number of myoclonic jerks
ii Latencies to myoclonic jerks
iii GTCS

Further, latency values were used to calculate seizure score using the following formula:

S = 1 − (control latency /drug seizure latency)

Kindling was considered to be complete when animals exhibited stage 5 seizure on two consecutive occasions. (score = 0 for control animals and 1 for animals with no seizure activity). Animals were monitored for 24 h to record any mortality.

2.9. Study groups

2.9.1. Estimation of serum level of sodium valproate

Group 1: Fluorescent tagged nanoparticles (300 mg/kg, i.p)
Group 2: Standard sodium valproate (300 mg/kg, i.p)
Group 3: Nano sodium valproate (~300 mg/kg of sodium valproate, i.p)

2.9.2. PTZ induced seizures

Group 1: Vehicle control
Group 2: PTZ (60 mg/kg, i.p)
Group 3: Standard sodium valproate (300 mg/kg, i.p)
Group 4: Nano sodium valproate (~300 mg/kg of sodium valproate, i.p)

2.9.3. PTZ induced kindling

Group 1: Vehicle control
Group 2: PTZ kindling (30 mg/kg, i.p)

Group 3: Sodium valproate (300 mg/kg, i.p)

Group 4: Nano sodium valproate (~150 mg/kg of sodium valproate, i.p)

Group 5: Nano sodium valproate (~75 mg/kg of sodium valproate, i.p)

2.10. Biochemical analysis

2.10.1. Collection of samples

At the end of the experiments, samples of brain tissue were collected after sacrificing the animals. All samples were washed with ice-cold saline, and stored at −80 °C till the time of analysis.

2.10.2. Tissue preparation

The whole brain of each rat was dissected out. 10% w/v tissue homogenate was prepared from each brain specimen using 0.1 M phosphate buffer kept maintaining ice-cold temperature and pH 7.4. Lipid peroxidation product and reduced glutathione were estimated.

2.10.3. Brain lipid peroxidation

Lipid peroxidation products were assessed using malondialdehyde (MDA) as an indicator with the method as reported previously (Ohkawa et al., 1979). Absorbance was measured at 532 nm using a spectrophotometer (Specord 200, Analytic Jena AG, Germany).

2.10.4. Measurement of reduced glutathione

Reduced glutathione was measured using protocol reported in literature (Ellman, 1959). Absorbance was taken at 412 nm within 15 min in a spectrophotometer (Specord 200, Analytic Jena AG, Germany).

2.11. Statistical analysis

Statistical analysis of the data was done using SPSS version 22 and R statistical computing environment (http://www.R-project.org) version 3.5.2. Results were expressed as mean ± SD. One way analysis of variance and Bonferroni post hoc tests were used to calculate any statistically significant difference among different groups. Kruskal- Wallis ANOVA was applied for Racine score as similar data analysis has been recently reported in literature (Van Erum et al., 2019).

3. Results

3.1. Characterization of nano sodium valproate

The size (mean ± SD) of the nanoparticles was 220 ± 78 nm with 192 nm as the mode value (Fig. 1A, Fig. 1**B**). The TEM image showed sphere shaped particles (Fig. 1C). Zeta potential was −32.9 mV.

3.2. Estimation of drug loading

Drug loading percentage was estimated to be 13.96% w/w and encapsulation efficiency was estimated to be 30%.

3.3. Drug release kinetics

In vitro drug release kinetics showed sustained release of drug from nanoparticles (Fig. 2). More than 85% of the loaded drug was released from nanoparticles by the end of 6th day.

3.4. Rhodamine loaded nanoparticles in brain

Study of sagittal sections of cerebral cortex under fluorescent microscope showed fluorescence indicating the presence of rhodamine loaded nanoparticles in brain, thus implying that nanoparticles crossed BBB. (Fig. 3A, B). No fluorescence was seen in the brain section of control rats while red fluorescence was seen in the brain section of rats administered with fluorescent tagged nanoparticles.

3.5. Estimation of serum level of sodium valproate

Sodium valproate level (expressed as mean ± SD) was measured in serum samples at 15 min, 1 h and 24 h **(**Table 1**).** Sodium valproate reached therapeutic levels in serum at 15 min and 1 h in both groups (standard sodium valproate 300 mg/kg, i.p. and nano sodium valproate ~300 mg/kg of sodium valproate, i.p.), but were undetectable at 24 h in both groups.

3.6. Effect in animal models of seizures

3.6.1. PTZ induced seizures

In PTZ induced seizures, nano sodium valproate (~300 mg/kg of sodium valproate i.p.) group showed significant ($p < 0.01$) protection at 6 h after drug administration which was not seen in standard sodium valproate (300 mg/kg i.p.) group (Table 2). There were 6 animals per group and no mortality was observed in any group.

3.6.2. PTZ induced kindling

In chronic model, significant protection was observed in nano sodium valproate groups at the doses equivalent to 150 mg/kg ($p < 0.01$) and 75 mg/kg ($p < 0.01$) of sodium valproate i.p. as compared to PTZ group. Standard sodium valproate group also showed complete protection in this model at the dose of 300 mg/kg i.p. ($p < 0.01$) (Table 3). There were 6 animals per group and no mortality in any group.

3.7. Effect on behavioral parameters

A significant difference in the performance of various tasks for cognitive and behavioral functions was observed amongst the groups.

3.7.1. Elevated plus maze test

In the PTZ induced seizures, nano sodium valproate (~300 mg/kg of sodium valproate i.p.) group showed significant decrease in retention transfer latency as compared to standard sodium valproate (300 mg/kg i.p.) group ($p < 0.001$) and PTZ group ($p < 0.001$) (Fig. 4A). In the PTZ induced kindling, standard sodium valproate (300 mg/kg i.p.) group and nano sodium valproate (~150 mg/kg and ~75 mg/kg of sodium valproate i.p.) groups showed significant ($p < 0.001$) decrease in retention transfer latency as compared to PTZ kindling group. However, there was no statistically significant difference amongst the drug treated groups and vehicle control group (6 animals per group) (Fig. 4B).

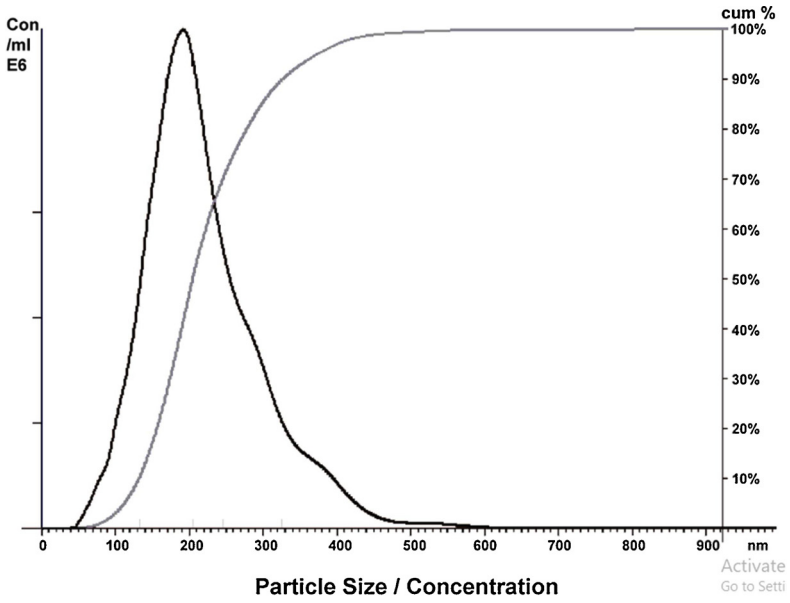
3.7.2. Passive avoidance test

In the PTZ induced seizures, nano sodium valproate (~300 mg/kg of sodium valproate i.p.) group showed significant increase in retention latency as compared to standard sodium valproate (300 mg/kg i.p.) group ($p < 0.01$) and PTZ group ($p < 0.01$) (Fig. 5A). In the PTZ induced kindling, standard sodium valproate (300 mg/kg i.p.) and nano sodium valproate (~150 and ~75 mg/kg of sodium valproate i.p.) groups showed significantly better retention latency as compared to PTZ kindling group ($p < 0.01$) although there was no statistically significant difference amongst drug treated groups and vehicle control group (6 animals per group) (Fig. 5B).

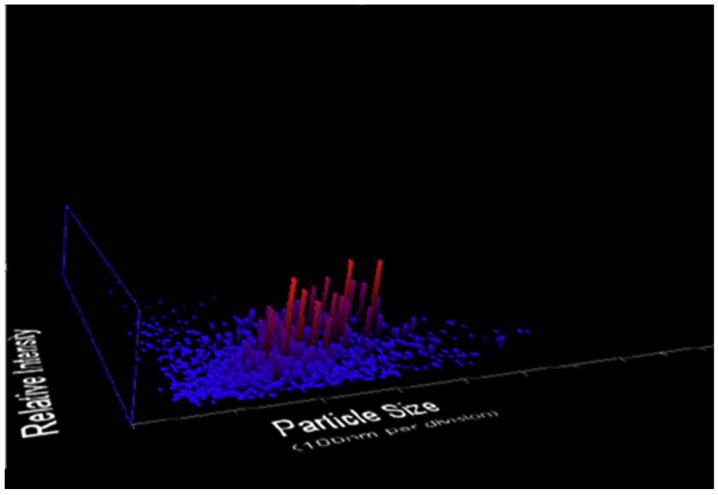
3.8. Effect on biochemical parameters

3.8.1. Measurement of brain lipid peroxidation

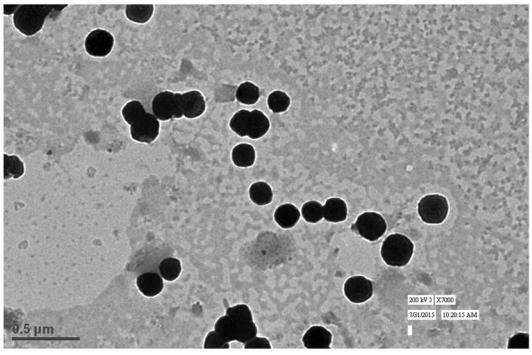
In PTZ induced seizures, brain MDA level an indicator of lipid peroxidation, was found to be significantly ($p < 0.01$) higher in PTZ group as compared to control group. It was significantly ($p < 0.01$) higher in standard sodium valproate group (300 mg/kg i.p.) as



A



B



C

Fig. 1. A. Size of lyophilized PLGA based nanoparticles estimated by dynamic light scattering. **B.** Distribution of lyophilized PLGA based nanoparticles estimated by dynamic light scattering. **C.** Electron microscopic pictures of drug loaded lyophilized nanoparticles at high resolution.

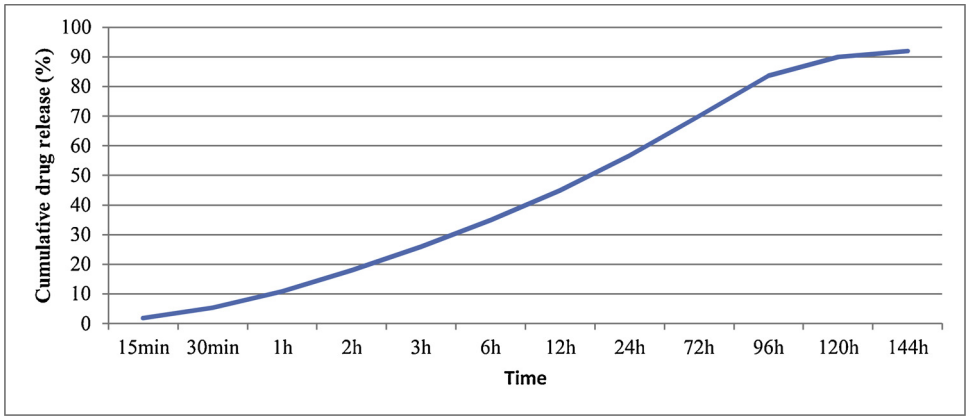


Fig. 2. Cumulative percentage of sodium valproate released from nanoparticles with time (h) evaluated with *in vitro* drug release assay.

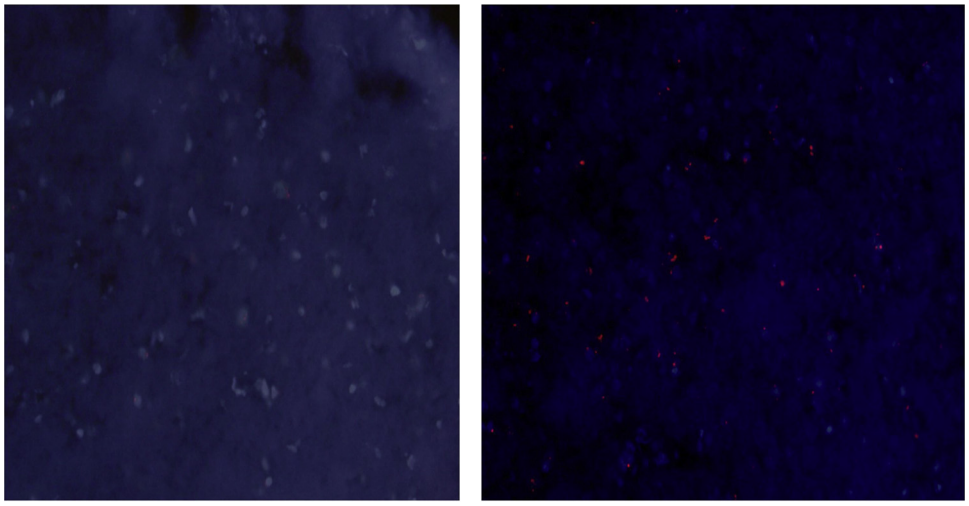


Fig. 3. A. Brain section of control animal at the level of cerebral cortex. **B.** Brain section of animal administered with fluorescent nanoparticles at the level of cerebral cortex. Arrows showing fluorescence.

Table 1
Concentration of sodium valproate (µg /ml) in serum of rats estimated by HPLC at 210 nm.

Drug Conc in µg/ml (mean ± SD)	At 15 min	At 1 h	At 24 h
Standard sodium valproate (300 mg/kg i.p.)	130 ± 8.0	128 ± 4.5	Not detected
Nano sodium valproate (~300 mg/kg of sodium valproate i.p.)	109 ± 8.4	70 ± 4.8	Not detected

compared to nano sodium valproate group (~300 mg/kg of sodium valproate i.p.) (Fig. 6A). In PTZ kindling model, brain MDA level was significantly higher in PTZ kindling group as compared to vehicle control group ($p < 0.01$), standard sodium valproate group (300 mg/kg i.p.) ($p < 0.01$) and nano sodium valproate groups (~150 and ~75 mg/kg of sodium valproate i.p.) ($p < 0.01$). No significant difference was observed amongst the drug treated groups and vehicle control group (6 animals per group) (Fig. 6B).

3.8.2. Measurement of reduced glutathione

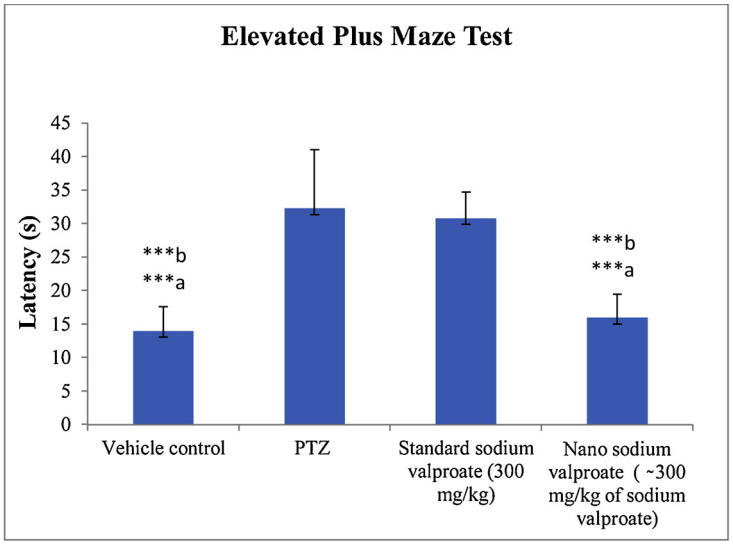
In PTZ induced seizures, brain GSH levels was found to be significantly decreased in PTZ group as compared to vehicle control group ($p < 0.001$). It was also significantly decreased in standard sodium valproate group (300 mg/kg i.p.) as compared to nano sodium

Table 2
Effects of nano sodium valproate in PTZ induced seizures. *p < 0.05, **p < 0.01 and ***p < 0.001. (n = 6); a- as compared to PTZ group, b- as compared to standard sodium valproate (300 mg/kg i.p.) group.

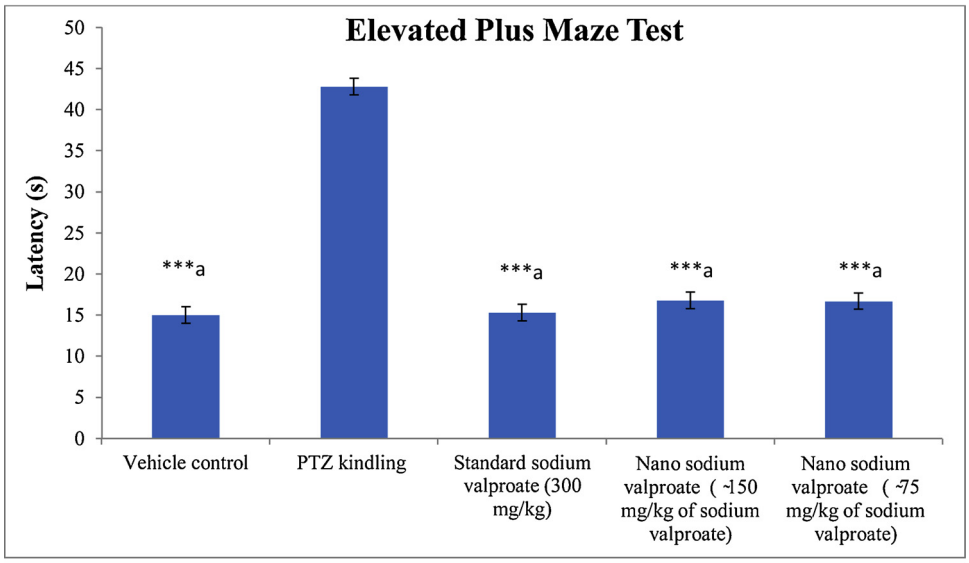
S. no.	Groups	Latency to myoclonic jerks (s)	GTCS with loss of righting reflex	Protection against seizures
1.	Vehicle control	–	–	–
2.	PTZ	16.67	Present	0/6
3.	Standard sodium valproate (300 mg/kg i.p.)	48.66	Present	0/6
4.	Nano sodium valproate (~300 mg/kg of sodium valproate i.p.)	723.00	Absent	4/6**a,**b

Table 3
Effects of nano sodium valproate in PTZ induced kindling. (n = 6); *p < 0.05, **p < 0.01 and ***p < 0.001, a- as compared to PTZ kindling group.

S. no.	Group	Protection against seizures	Racine score
1.	Vehicle control	–	–
2.	PTZ kindling	0/6	0
3.	Standard sodium valproate (300 mg/kg i.p.)	6/6**a	6
4.	Nano sodium valproate (~150 mg/kg of sodium valproate i.p.)	6/6**a	6
5.	Nano sodium valproate (~75 mg/kg of sodium valproate i.p.)	6/6**a	6



A



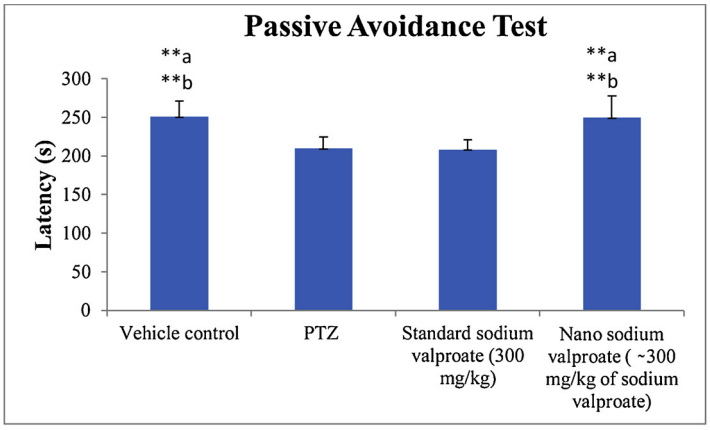
B

Fig. 4. A. Effect of nano sodium valproate (~300 mg/kg of sodium valproate i.p) on retention transfer latency in elevated plus maze test in PTZ induced seizures. (n = 6); *p < 0.05, **p < 0.01 and ***p < 0.001 a- as compared to PTZ group; b- as compared to standard sodium valproate group. **B.** Effect of nano sodium valproate (~150 and ~75 mg/kg of sodium valproate i.p) on retention transfer latency in elevated plus maze test in PTZ induced kindling. (n = 6); *p < 0.05, **p < 0.01 and ***p < 0.001, a- as compared to PTZ kindling group.

valproate group (~300 mg/kg of sodium valproate i.p.) ($p < 0.01$) (Fig. 7A). In PTZ induced kindling, significant decrease was observed in brain GSH levels in PTZ kindling group as compared to drug treated groups i.e., standard sodium valproate (300 mg/kg) group and nano sodium valproate (~150 and ~75 mg/kg of sodium valproate) groups ($p < 0.001$). However, no significant differences were observed among drug treated groups and vehicle control group (6 animals per group) (Fig. 7B).

4. Discussion

Conventional therapy of epilepsy includes drugs such as sodium valproate, phenytoin, carbamazepine, levetiracetam, clobazam and others. These drugs are widely used due to their time proven efficacies.



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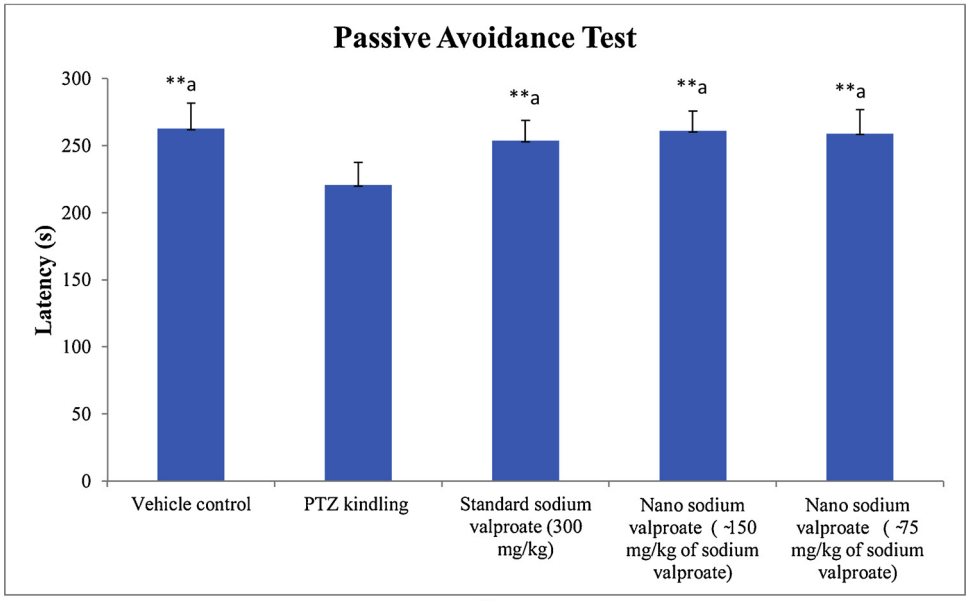

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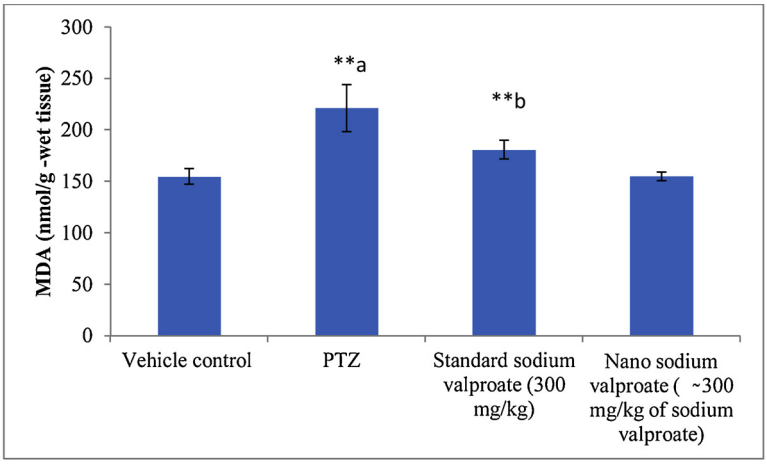
Fig. 5. A. Effect of nano sodium valproate (~300 mg/kg of sodium valproate i.p.) on retention latency in passive avoidance test in PTZ induced seizures. (n = 6); *p < 0.05, **p < 0.01 and ***p < 0.001. a- as compared to PTZ group; b- as compared to standard sodium valproate (300 mg/kg i.p.) group. **B.** Effect of nano sodium valproate (~150 and ~75 mg/kg of sodium valproate i.p.) on retention latency in passive avoidance test in PTZ induced kindling. (n = 6); *p < 0.05, **p < 0.01 and ***p < 0.001; a- as compared to PTZ kindling group.

Clinical experience with these drugs is extensive. But there are certain limitations with these drugs such as side effects, drug–drug interactions, drug resistance and high dose requirement (Johannessen and Johannessen, 2010; St. Louis et al., 2009; Stapleton et al., 2008).

This has prompted medical science to articulate a multimodal approach in its endeavor towards management of this disease. Application and expansion of traditional knowledge about herbal remedies and exploration of newer drug delivery systems for epilepsy is an integral part of this approach. There has been much research done to improve epilepsy management with natural herbs and plants such as curcumin (Reeta et al., 2009), *Zizyphus jujube* (Pahuja et al., 2012), *Anacyclus pyrethrum* (Pahuja et al., 2013), Panchgavya Ghritam (Joshi et al., 2015) *etc.* These natural remedies provide certain advantages such as reduction in the doses of antiepileptic drugs and dose related side effects.

Another approach currently under extensive research is formulation of novel drug delivery systems using nanotechnology (Jaiswal et al., 2010). Using this idea, we formulated stable PLGA based, polysorbate 80 coated, sodium valproate loaded nanoparticles. Previous works done on PLGA nanoparticles showed them to have good stability, biodegradability, biocompatibility and reduced immunogenicity in addition to resistance to phagocytosis by blood macrophages (Gulati et al., 2010; Makadia and Siegel, 2011; Muthu et al., 2011; Wong et al., 2012). Our particles also exhibited good stability with slow and sustained drug release pattern in *in vitro* drug release assay and these effects were reflected in chronic animal model of seizure.

Rhodamine, like sodium valproate is a ligand for p-glycoprotein with very low capability to cross brain capillary endothelial cells. It is known from previous studies that the efflux of sodium valproate (carried by efflux pump proteins such as p-glycoproteins) is 2.7 fold greater than influx (Varshosaz et al., 2011). In this study, we examined the penetration and distribution of rhodamine loaded nanoparticles, using fluorescence microscope, in brain at single time point of 6 h and observed fluorescence in cortex region of rat brain. This was an indirect demonstration that sodium valproate loaded nanoparticles also crossed BBB. The suggested mechanism for increased permeability of polysorbate 80 surface modified nanoparticles was its adsorption on plasma lipoproteins primarily apolipoproteins E, B and also A-I in blood and interaction with low density lipoprotein (LDL) receptors present on BBB endothelial cells. Lipoprotein coated nanoparticles are taken up by



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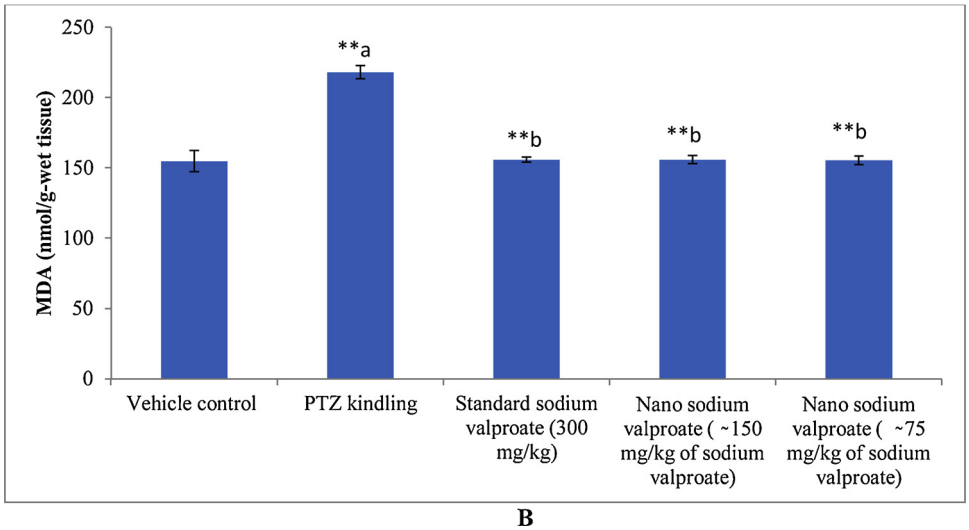


B

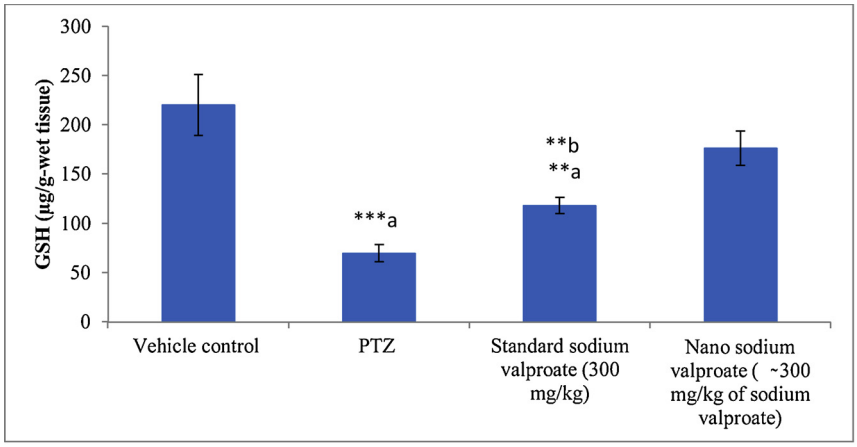
Fig. 6. A. Effect of nano sodium valproate (~300 mg/kg of sodium valproate i.p.) on brain MDA level in PTZ induced seizures. (n = 6); *p < 0.05, **p < 0.01 and ***p < 0.001. a- as compared to vehicle control group; b- as compared to nano sodium valproate (~300 mg/kg of sodium valproate i.p.) group. **B.** Effect of nano sodium valproate (~150 and ~75 mg/kg of sodium valproate i.p.) on brain MDA levels in PTZ induced kindling. (n = 6); *p < 0.05, **p < 0.01 and ***p < 0.001. a- as compared to vehicle control group; b- as compared to PTZ kindling group.

scavenger receptors SR-BI located on BBB endothelium and receptor-mediated endocytosis helps in concentrating the number of nanoparticles in brain and prolonging their action (Georgieva et al., 2014; Kreuter et al., 2002; Vergoni et al., 2009). Apart from receptor-mediated endocytosis, other mechanisms such as transcytosis have been proposed to explain the entry of nanoparticles into brain (Barbu et al., 2009; Bhaskar et al., 2010; Patel et al., 2012). Hence, it could be postulated that encapsulation of drug in targeted nanoparticles might be helping it to overcome p-glycoprotein induced barrier (Bhaskar et al., 2010; Potschka et al., 2002; Reimold et al., 2008; Vergoni et al., 2009).

Sodium valproate was quantified in rat serum at definite time intervals following i.p. administration of nano sodium valproate and standard sodium valproate. When compared to standard sodium valproate group, nanoparticles group attained lower serum concentration of sodium valproate. This may be due to rapid degradation of up to 30% of PLGA nanoparticles in initial stages followed by slow degradation and delayed clearance of rest 70% (Makadia and Siegel, 2011). It might also be related to prolongation of effect of sodium valproate loaded nanoparticles. It is also known from previous studies that nanoparticles administered via i.p. route have increased possibility of phagocytosis by peritoneal macrophages. To some extent we tried to overcome this obstacle by using PLGA based polymer and coating by polysorbate 80 as suggested by Makadia and Siegel, 2011.

It was found that nano sodium valproate was effective till 6 h of its administration in acute PTZ model as compared to standard sodium valproate which showed complete protection till 2 h at comparable doses. The prolongation of effect may be due to sustained release of drug and effective brain targeting (Yusuf et al., 2012; Vergoni et al., 2009).

In kindling model the efficacy of lowest dose of nano sodium valproate used in our study was comparable to the standard dose of sodium valproate. We attribute this improvement to a slow and sustained drug release from nanoparticles which effectively targeted brain by evading phagocytosis and degradation in circulation. A progressive accumulation of the nanoparticles along with biodegradable, biocompatible and functionalization properties, and flexible surface modification (for example, coating by polysorbate 80) led to prolonged efficacy of nano sodium valproate at reduced dosages (Barbu et al.,



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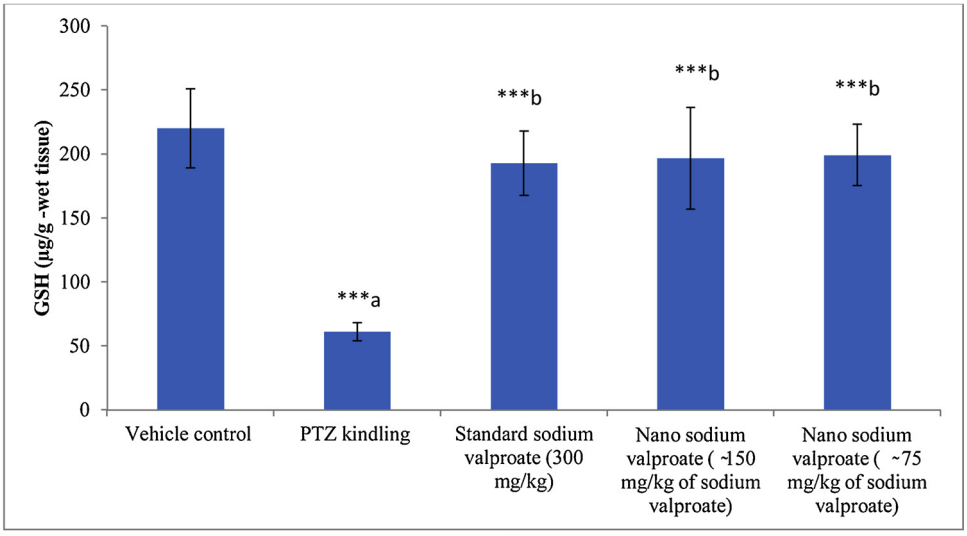

B

Fig. 7. A. Effect of nano sodium valproate (~300 mg/kg of sodium valproate i.p.) on brain GSH levels in PTZ induced seizures. (n = 6); *p < 0.05, **p < 0.01 and ***p < 0.001. a- as compared to vehicle control group; b- as compared to nano sodium valproate (~300 mg/kg of sodium valproate i.p.) group. **B.** Effect of nano sodium valproate (~150 and ~75 mg/kg of sodium valproate i.p.) on brain GSH levels in PTZ induced kindling. (n = 6); *p < 0.05, **p < 0.01 and ***p < 0.001. a- as compared to vehicle control group; b- as compared to PTZ kindling group.

2009).

In this study, we found significantly less behavioral impairment in elevated plus maze and passive avoidance tests in nano sodium valproate as compared to standard sodium valproate and PTZ groups in acute seizure model while in chronic model, protection against behavioral impairment was seen in both nano sodium valproate and standard sodium valproate groups. The lack of adverse effect on animal behavior could be attributed to protection from seizures. Studies had demonstrated significant behavioral impairment due to seizures in animals and protection from seizures led to less behavioral impairment (Reeta et al., 2011; Wu et al., 2016; Zhu et al., 2018). Although, effect of nanoparticles on behavior and cognition is currently debatable, some studies did not find cognitive and behavioral impairment with nanoparticles (Pahuja et al., 2015; Saraiva et al., 2016; Xie et al., 2012). With our study design and results available, it was difficult to attribute these effects to either nano-formulations or protection from seizures precisely. Furthermore, in this study cognitive functions were evaluated using elevated plus maze and passive avoidance tests. Although elevated plus maze test is commonly used for anxiety assessment, its application in learning and memory evaluation in rodents has been documented in literature and research in the field of nootropic drugs. This test has advantages such as lack of aversive stimulus, simple and less time consuming procedure (Blatt and Takahashi, 1998; Da Cunha et al., 2005; Itoh et al., 1990; Kumar et al., 2007a; Sharma and Kulkarni, 1992; Sonkusare et al., 2005). The idea is that if the animal has had a prior exposure to closed and open environmental conditions as devised in elevated plus maze, it will learn to avoid open environment and the transfer latency (time taken by the animal to enter closed arm from open arm in elevated plus maze) is likely to reduce. Thus, the change in latency time implicates memory function of the animal. The complementary test is passive avoidance task, which was also used in this study. In this test, the animal is exposed to aversive stimulus in the form of electric shock. On repetition of same stimulus, the animal learns to stay in the lit chamber and avoids the dark chamber where the animal was exposed to the foot shock. In this way, the animal acts against its nature of preferring dark enclosed territory in favor of its prior experience based learning. Thus, this test is also applicable in memory assessment of the rodents wherein it learns and recalls prior experiences about the environment and relates it with the aversive stimulus (Rodriquiz and Wetsel, 2006).

In this study, PTZ group showed significant oxidative stress compared to normal rats. It was observed that nano sodium valproate ameliorated the oxidative stress by preservation of brain GSH and preventing lipid peroxidation in both acute and chronic seizures models. This protective effect was not seen with standard sodium valproate in acute model, but was definitely present in chronic model.

Moreover, there was no statistically significant difference in brain MDA and GSH levels among standard sodium valproate and nano sodium valproate groups suggesting their comparable protective effect against oxidative stress (Aguiar et al., 2012). Although nanoencapsulation has been shown to reduce oxidative damage (Benvegnú et al., 2012), we infer that protection against seizures is probably the reason behind attenuated oxidative stress in sodium valproate and nano sodium valproate groups compared to PTZ group in our study (Ribeiro et al., 2005; Saraiva et al., 2016; Wang et al., 2014; Yusuf et al., 2012).

5. Conclusion

BBB is an obstacle for CNS therapeutics and has led to failure of many promising drug candidates. In our study, we explored the possibility of nanoparticles engineered to overcome this barrier. In this study, nanoparticles of sodium valproate were formulated and evaluated in animal models of epilepsy. We concluded that when compared to conventional therapy, polysorbate 80 decorated sodium valproate loaded PLGA based nanoparticles showed efficacy at reduced doses and for a longer duration of time in experimental models of epilepsy. Thus, nanoparticles based therapy may provide better treatment options for epilepsy.

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Declaration of Competing Interest

None.

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