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Preparation of Bacoside A encapsulated PLGA-PEG nanoparticles for neuroprotection against kainic acid-induced excitotoxicity

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Abstract

The inherent challenges posed by the blood-brain barrier (BBB) complicate the effective delivery of neuroprotective drugs. In response to these limitations, plant-based nanoparticle formulations are gaining interest for enhancing patient outcomes while minimizing side effects. Bacoside A (BM3) is a nootropic and neuroprotective saponin found in *Bacopa monnieri*. Owing to its limited permeability across the blood-brain barrier, BM3 is encapsulated within polymeric nanoparticles (NPs) for effective delivery. This study investigates the effects of BM3 encapsulated PLGA-PEG nanoparticles (BM3NPs) on kainic acid (KA)-induced excitotoxicity in a mouse model. It evaluates the protective effects of BM3NPs against neuroinflammation, oxidative stress, overexpression of seizure markers, and dysregulation of the mTOR pathway. BM3NPs exhibited an optimal size of 165.5 nm and a zeta potential of -32.5 mV, ensuring effective drug delivery. TEM studies demonstrated that BM3NPs (4 mg/kg, b.w.) reduced KA-induced brain tissue damage by restoring normal nuclear outline and strengthening brain membrane integrity. BM3NP also suppressed the overexpression of AMPA glutamate receptors and mTORC1 signaling. BM3NP treatment also led to an increase in antioxidant levels while reducing the expression of proinflammatory cytokines. Overall, the findings suggest that BM3NPs could serve as a promising therapeutic option for addressing KA-induced excitotoxicity.

1. Introduction

Bacoside A (BM3) is a bioactive compound found in the herb *B. monnieri*, which has been used in traditional Ayurvedic medicines for centuries. This compound has gained significant attention in recent years for its potential health benefits, particularly its effects on cognitive function and mental health. Numerous studies have been conducted to explore the neuroprotective effects of bacoside A. Bacoside A has been found to prevent age-related cognitive decline and support memory and learning abilities. The significance of the compound lies in its potential to offer natural alternatives for cognitive enhancement and mental health support. Bacoside A consists of four triterpenoid saponins, *viz.*, bacoside A3, bacopaside II, jujubogenin isomer of bacopasaponin C (bacopaside X) and bacopasaponin C (Deepak et al. 2005). Administration of *B. monnieri* and bacoside A resulted in an enhancement of GABA-mediated inhibition in rats with epilepsy (Mathew et al. 2012).

Epilepsy is a neurological condition characterized by spontaneous and recurrent seizures. One key factor in epileptic seizures is the abnormal synchronization of neurons, mediated by ionotropic glutamate receptors. The mammalian Target of Rapamycin (mTOR) dysregulation has been associated with various genetic and acquired forms of epileptogenesis. Targeting AMPA receptors and the mTOR pathway may represent a potential therapeutic strategy for managing epilepsy. CX3CL1/ fractalkine, FOXO3a, HMGB1, and pro-inflammatory cytokines (IL-1β, IL-6, IL-8 etc) act as potential indicators of brain inflammation among patients with epilepsy. Current antiepileptic drugs (AEDs) provide symptomatic relief but have harmful side effects. Also, the efficacy of treatment measures is compromised by the limited ability of therapeutic molecules to traverse the BBB. Polymeric nanoparticles have emerged as a promising solution to address the limitations associated with conventional drug delivery systems. These

nanoparticles offer the advantage of controlled drug release, enabling precise targeting of specific sites within the body. Additionally, they exhibit excellent biodegradability, further enhancing their potential as effective drug carriers. The purpose of this study was to investigate the effects of bacoside A encapsulated PLGA-PEG nanoparticles (BM3NP) on KA-induced excitotoxicity in a mouse model. The study aimed to evaluate seizure scores and the anti-seizure effects of BM3NP. It focused on how BM3NP improves efficacy compared to free BM3. The research examined mRNA and protein expression of AMPA glutamate receptor subunits, proteins in the mTOR pathway, seizure biomarkers, inflammatory cytokines, oxidative stress parameters, brain membrane integrity, and conducted histopathological analyses.

2. Materials and methods

2.1. Chemicals

Nanoparticles were synthesized using the biodegradable polymer PLGA-b-PEG (50:50) from Sigma Aldrich, St. Louis, MO, USA. KA was procured from Merck, Germany and standard bacoside A from Sigma Aldrich, St. Louis, MO, USA. All other chemicals used were of analytical quality and were obtained from verified vendors.

2.2. Preparation of PLGA-PEG encapsulated nanoparticles

BM3 was isolated from *B. monnieri* methanol extract, purified, and characterized by co-HPTLC with its authentic standard. The nanoprecipitation (solvent displacement) technique was followed to prepare PLGA-b-PEG encapsulated nanoparticles (BM3NPs) and the methodology outlined by Sekhar et al. was followed (Şimşek et al. 2013; Sekhar et al. 2023).

2.3. Release profile of nanoparticles

UV/Vis spectrometer (UV 1650 PC, Shimadzu, Japan) was used to measure the absorption and transmission of light by the nanoparticles for determining encapsulation efficiency of BM3. Colloidal suspension of nanoparticles was centrifuged at 14000 rpm at 4°C, absorbance of the supernatant was recorded and the amount of entrapped BM3 was calculated according to the equation EE% = (amount of entrapped drug/total drug added ×100) (Sekhar et al. 2023).

By using the dialysis technique, the release profile of BM3 from BM3NP nanoparticles was examined. A dialysis bag was packed with a colloidal suspension of BM3NP (1 mL) equal to 1 mg of BM3 and placed within 50 mL of phosphate buffered saline (PBS). UV spectroscopy was used to measure the release of BM3 at regular intervals. Percentage release of BM3 is calculated as Release (%) = $[BM3NP]_{rel}/[BM3NP]_{tot}$ x 100, where $[BM3NP]_{rel}$ is the concentration of released BM3 collected at time t and $[BM3NP]_{tot}$ is the total quantity of BM3 entrapped in nanoparticles (Sekhar et al. 2023).

2.4. *In vivo* studies

2.4.1. Animals

Healthy Swiss albino male mice (8–14 weeks old, 20–25 g weight) were used for the experiments. They were kept in polyurethane cages (three in each) at an ambient temperature of 25 ± 2°C and a relative humidity of 50.5% ± 5, and a constant light-dark schedule (12 h light and 12 h dark cycle) with *ad libitum* access to both standard rodent food and water. The Institutional Animal Ethics Committee (IAEC) of Jawaharlal Nehru Tropical Botanic Garden and Research Institute has approved the experimental procedures (No. B1/05/2016/PPD-02), which were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. All animal experimental procedures were carried out according to the ISO 10993 and OECD guideline 2019.

2.4.2. Experimental design

Animals were assigned to five treatment groups randomly (n = 6 for each group), *viz.*, (1) control, saline-treated mice (Con), (2) KA-treated mice (KA), (3) BM3 and KA-treated mice (BM3 + KA), (4) BM3NP and KA-treated mice (BM3NP + KA), (5) LEV and KA-treated mice (LEV + KA). KA was given to the mice to induce epileptic seizures at dose of 10 mg/kg, b.w. (i.p) (Albala et al. 1984; Liu et al. 2010). Controls received the same treatment except for KA that was substituted by an equal volume of saline. After 10 mins, the administration of BM3, BM3NP and LEV was made intravenously in the respective groups at dose level 4 mg/kg, b.w. (Marini et al. 2004). After KA injections, behavioural alterations and seizure severity of mice were scored continuously for 3 h using modified Racine's scale (Racine et al. 1972): Grade 0 - no abnormal movements; Grade 1 - head bobbing, tremor, backward walking, wet dog shake; Grade 2 - intermittent forepaw myoclonus, rearing, and falling; Grade 3 - clonic convulsions; Grade 4 - tonic-clonic generalized convulsions and Grade 5 - status epilepticus (SE). After 3 hours, mice were sacrificed by cervical dislocation. The brain was rapidly dissected to evaluate oxidative stress parameters, brain membrane permeability studies, mRNA expression studies and Western blotting according to the standard protocols (Kunjumon et al. 2022).

2.4.3. Ultrastructural morphology analysis

Brain tissues from control, KA and BM3NP + KA treated groups were evaluated for ultra-morphological changes using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Kunjumon et al. 2021b).

2.4.4. Short-term toxicity evaluation in mice

BM3 and BM3NP were administered intravenously at a dose of 20 mg/kg, b.w. daily for 14 days. Normal saline was administered to the control group. Control and treated (BM3/BM3NP) groups were observed daily. Clinical observations include respiratory, motor, convulsion, reflexes, ocular signs, cardiovascular signs, salivation, piloerection, analgesia, muscle tone, gastrointestinal and skin changes. The experimental procedure was carried out in accordance with the ISO 10993 and OECD guideline 2019. On the 15th day, all mice were sacrificed through cervical dislocation. Biochemical and hematological

parameters were estimated. The gross pathology of various organs, including the brain, liver, kidney, heart, and spleen, were examined for visible morphological abnormalities before tissue fixation. Histopathological evaluation of tissues of all groups (control, BM3 and BM3NP) were also conducted (Viswanathan et al. 2014).

2.4.5. Studies on oxidative stress parameters

Swiss albino male mice (20–25 g) were selected and divided into seven groups: Group 1 - control, Group 2 - KA (10 mg/kg, b.w.), Group 3 - BM3 (1) 4 mg/kg, b.w. + KA, Group 4 - BM3NP (1) 4 mg/kg, b.w. + KA, Group 5 - BM3 (2) 8 mg/kg, b.w. + KA, Group 6 - BM3NP (2) 8 mg/kg, b.w. + KA, Group 7 - LEV (4 mg/kg, b.w.) + KA. Mice brain homogenate (10%) was used to determine total antioxidant capacity (ab65329, Abcam), lipid peroxidation (Ohkawa et al. 1979), antioxidant enzymes like glutathione-S-transferase, superoxide dismutase, glutathione reductase levels and reduced glutathione levels (Moron et al. 1979; Ishigami 1998; Viswanathan et al. 2014), nitric oxide level (Ignarro et al. 1987) and H_2O_2 concentration (ab138874, Abcam) (Lee et al. 2022).

2.4.6. Determination of brain permeability and membrane integrity

Brain membrane permeability and integrity after BM3/BM3NP + KA was evaluated using the Evans blue (EB) staining assay. Mice were given EB intravenously at a dose of 80 mg/kg, b.w. After 12 hours, the brains of the mice were taken out, weighed and homogenized in PBS (pH 7.4). TCA (2.5 mL) was added to the samples, and the mixture was kept on ice for 30 min. Samples were then centrifuged at 1000 x g, 4°C for 30 min, and the absorbance(s) of the supernatants were measured at 610 nm (Kunjumon et al. 2022).

2.4.7. Protein expression study using ELISA method

Fractalkine/CX3CL1 Mouse ELISA kit (Thermo Scientific, EMCX3CL1) was used to determine the protein concentration, and the procedure was performed according to the instructions provided in the kit.

2.4.8. Semi-quantitative mRNA expression studies of seizure markers

Brain tissue homogenate was used to extract total mRNA as per the manufacturer's protocol using Trizol reagent. mRNA (600 ng) was used for the cDNA synthesis in a reaction volume of 20 µL using iScript™ cDNA Synthesis Kit (Bio-Rad). The primers designed are given in Table S1. Real-time PCR reaction was carried out with TB Green® Premix Ex Taq™ II (Tli RNase H Plus) of total reaction volume (20 µL) and 40 cycles were run as per the protocol. GAPDH and beta actin (β actin) were used as housekeeping genes. Relative mRNA expression was determined using the comparative $2^{-\Delta\Delta Ct}$ method (Sekhar et al., 2023).

2.4.9. Protein expression analysis by Western blot

After BM3/BM3NP + KA treatment, brain tissues were removed immediately and then homogenized in RIPA buffer containing protease inhibitor cocktail by incubating in ice for 30 min. Samples were homogenized and centrifuged at 12,000 rpm for 10 min. Supernatant was used to estimate protein concentrations with the Bradford reagent. Samples were boiled for 5 min and microcentrifuged for 5 min. Equal quantities of proteins (10 µg) were resolved using electrophoresis. Gel was then transferred onto a polyvinylidene difluoride membranes (Thermo Fisher). Target proteins were detected by chemiluminescence by SignalFire" ECL Reagent (#6883, Cell Signaling Technology, USA). Antibodies used and their dilutions are given in Table S2 (Sekhar et al. 2025).

2.5. Statistical analysis

A one-way analysis of variance (ANOVA) was conducted using SPSS 10 software to compare the parameters in the *in vitro* and *in vivo* studies followed by Tukey HSD post hoc test for comparing differences between the groups. Quantitative data was presented as the mean ± standard deviation (SD) of three independent experiments and analyzed by an independent sample student's t-test. Level of significance was kept at $p < 0.05$.

3. Results

DLS spectrum showed the average particle size of BM3NP as 165.5 nm (Fig. 1a). BM3NP depicted a zeta potential of -32.5 mV and polydispersity index (PDI) value of 0.04 (Fig. 1b). PDI value represents the homogenous nature of a sample, values from 0.0–1.0 denote the uniformity of the dispersed particles and a good delivery system (Sorasitthiyanukarn et al. 2018; Kunjumon et al. 2021a, b).

3.1. Release kinetics of BM3NP

For the release study, dialysis was carried out with BM3NPs in 1 L of PBS (pH 7.4) at 37°C using a 10 kDa cutoff dialysis membrane. Amount of BM3 released from the NPs was measured spectrophotometrically at 278 nm. Controlled release of BM3 molecules from the co-polymer was evident in the release kinetics of BM3NPs. After 15 h of dispersion in phosphate buffer at a physiological pH, 50% of the molecules were released (Fig. 1c). Thereafter, a sustained release of BM3 was detected over time. This release profile infers that BM3NPs are efficient drug delivery systems with reduced drug leakage. Encapsulation efficiency was calculated as 90%.

3.2. Ultrastructural morphology analysis

SEM analysis was done to analyze the tissue distribution and localization of spherical shaped BM3NP nanoparticles in the mice brain (Fig. 2A and B). Non-spherical particles are susceptible to segregation and there is no segregation of nanoparticles in the present study. SEM images have confirmed the uniform distribution and homogenous nature of nanoparticles in the brain surface. The chemical characterization using TEM analysis of BM3NP shows spherical shape and character of nanoparticles (Fig. 2C). Present study depicts a similar brain architecture in BM3NP + KA treated mouse compared to

that of control using TEM analysis (Fig. 2D, E, and F). It shows the neuroprotective potential of BM3NP against KA excitotoxicity in mice. Therapeutic application of BM3NP effectively mitigated KA-induced damage and restored the ultrastructural organization of brain, including regular myelinated neurons (Fig. 2F). In the brain tissue images of the control group (Fig. 2D), regular nuclear outlines are indicated by the black arrow, while myelinated neurons are indicated by the yellow arrows. KA group (Fig. 2E) showed distorted endothelial cells with cytoplasmic swelling and severe neural oedema. BM3NP + KA group showed intracellular localization of BM3NP as electron-dense nanoparticles (highlighted in yellow circles).

3.3. Short-term toxicity evaluation

Current investigation showed no adverse changes in physical, biochemical, and hematological parameters throughout the study period of 14 days in BM3/BM3NP treated groups. There was no significant change in the mean body weight of the animals in BM3/BM3NP treated groups as compared to control group at the end of treatment.

No sign of local tissue damage was observed at the site of injection. There were no significant changes seen in total protein level in all the groups as compared to control group. No significant increase was observed in serum glucose, albumin, creatinine, bilirubin, total cholesterol, triglycerides and ALT activities in all the treated groups as compared to the control group (Table 1). However, BUN was found slightly elevated and AST level decreased in the treated group but within the reference values (Table 1) (Tamta et al. 2009). No significant changes were observed in any of the hematological parameters (Table 2) in the BM3/BM3NP-treated groups compared to the control group. These data suggest that BM3/BM3NP is a safe therapeutic choice.

Table 1
Short-term toxicity evaluation of BM3/BM3NP on biochemical parameters in the mice blood.

Biochemical Parameters	Control	BM3	BM3NP
Glucose (mg/dL)	96.96 ± 0.01	121.96 ± 0.01	119.31 ± 0.01
Protein (g/dL)	3.55 ± 0.02	4.07 ± 0.01	3.66 ± 0.01
Albumin (g/dL)	2.91 ± 0.01	2.64 ± 0.01	2.85 ± 0.01
Creatinine (mg/dL)	0.50 ± 0.01	0.76 ± 0.01	0.79 ± 0.01
BUN (mg/dL)	13.73 ± 0.01	31.11 ± 0.06	26.51 ± 0.03
Bilirubin (mg/dL)	0.04 ± 0.00	0.20 ± 0.01	0.27 ± 0.00
AST (IU/L)	227.55 ± 0.01	231.78 ± 0.02	157.07 ± 0.01
ALT (IU/L)	68.94 ± 0.01	65.66 ± 0.02	59.82 ± 0.01
Triglycerides (mg/dL)	88.46 ± 0.01	78.20 ± 0.01	68.37 ± 0.00
Total cholesterol (mg/dL)	99.27 ± 0.01	69.95 ± 0.01	77.50 ± 0.01

Values are expressed as mean ± SD; n = 6; BUN, Blood urea nitrogen; AST, Aspartate transaminases; ALT, Alanine transaminases; $p < 0.05$ *vs* control group.

Table 2
Short-term toxicity evaluation of BM3/BM3NP on hematological parameters in the mice blood.

Hematological Parameters	Control	BM3	BM3NP
Hb (g/dL)	15.6 ± 0.2	15.7 ± 0.1	14.33 ± 0.35
Total RBC count (x 10^5 / mm3)	24.66 ± 0.57	27.33 ± 2.08	28 ± 2.00
Total WBC count (x 10^3 / mm3)	3.51 ± 1.52	4.0 ± 1.0	3.88 ± 1.52

Values are expressed as mean ± SD; n = 6; Hb, hemoglobin; $p < 0.05$ *vs* control group.

3.4. Gross and histopathological evaluation of tissues

Histopathological analysis showed clear evidence of absence of toxicity in any of the organ in BM3/BM3NP treatment groups compared to control group. These are the findings of short-term toxicity BM3/BM3NP treatment for 14 days.

The study found that both BM3/BM3NP treated groups showed normal brain architecture (Fig. 3aii and iii). Control mouse liver sections showed normal liver architecture (Fig. 3 bi). Also, BM3 and BM3NP treated liver sections showed healthy hepatocytes and regeneration (Fig. 3bii and iii). Control and BM3/BM3NP treated kidney sections showed normal renal cortex and healthy glomeruli (Fig. 3ci, ii and iii)), likewise control and BM3/BM3NP treated groups showed no abnormalities in heart muscle cells,

myocardial fibers, or cardiac tissues (Fig. 3di, ii and iii). BM3/BM3NP treated groups exhibited a well-defined spleen section with normal capsule and lymphoid tissue (Fig. 3eii and iii). The results indicate that BM3NP is safe and there are no signs of tissue abnormalities.

3.5. Studies on oxidative stress parameters

Treatment of BM3/BM3NP enhanced the total antioxidant capacity in the brain tissues compared to KA control group. Similarly, the injection of 8 mg/kg, b.w. of BM3NP improved the total antioxidant capacity of brain tissues from 11.88 nmol (4 mg/kg, b.w. treatment group) to 12.5 nmol (Fig. 4a). MDA was found to be significantly increased in KA only group compared to control. MDA level was significantly decreased after BM3/BM3NP (4 mg/kg, b.w.) + KA treatment (19.21 ± 0.00 and 19.71 ± 0.01 μmol/mg protein, respectively) (Fig. 4b). KA exposure produced a very low level of GPx activity (0.32 ± 0.00 U/mg protein) in the brain tissues. Interestingly, the nanoencapsulation of BM3 (BM3NP) enhanced GPx activity 9-fold (9.71 ± 0.01 U/mg protein) (Fig. 4c). BM3NP (4 mg/kg, b.w.) administration was most effective in retaining SOD value (29.83 U/mL) to near normal control (Fig. 4d). It is evident that GR activity increased significantly in both 4 mg/kg, b.w. (4.25 ± 0.00 U/mg protein) and 8 mg/kg, b.w. (4.27 ± 0.00 U/mg protein) of BM3NP treatments (Fig. 4e).

KA induced high level of nitric oxide is reduced by the treatment of BM3 and BM3NP. Maximum reduction in NO level (113.3 ± 0.03 μM) was observed during the treatment with BM3NP (4 mg/kg, b.w.) which matched with the normal NO level of control group (Fig. 4f). Brain levels of glutathione significantly increased after 8 mg/kg, b.w. of BM3NP treatment (5.62 ± 0.00 nmol/mg protein) (Fig. 4g). Values showed a significant increase in H_2O_2 scavenging activity (%) in the group treated with BM3NP 4 mg/kg, b.w., i.v. (25.73 ± 0.02) and 8 mg/kg b.w., i.v. (41.36 ± 0.01) when compared to the KA only group (Fig. 4h).

3.6. Seizure score

Seizure activity was rated according to the Racine's scale. Racine convulsion scale scores of all groups are shown in Fig. 5a. Score was significantly higher in the KA group than the control group and significantly lower in the BM3NP + KA (4 mg/kg, b.w.) group.

3.7. Evaluation of brain membrane permeability and integrity

Treatment of KA at a dose of 10 mg/kg, b.w. showed a significant increase in the permeability of the brain membrane as indicated by the presence of Evans blue marker dye in brain tissues. However, administering BM3/BM3NP at a dose of 4 mg/kg, b.w. protected against the increased permeability caused by KA.

3.8. Expression of fractalkine/CX3CL1

CX3CL1/fractalkine, participates in inflammatory responses and increases during seizures. Fractalkine level is determined through ELISA method. Current results showed that BM3NP (4 mg/kg, b.w.) treatment decreased fractalkine expression against KA induced excitotoxicity model in mice.

3.9. mRNA expression studies of seizure markers in vivo

KA treatment alone induces an increase in the epileptic markers like fractalkine, HMGB1, FOXO3a and APP in mice brain tissues. Administration of BM3NP (4 mg/kg, b.w.) reduced the over-expression of epileptic markers and re-established normal expression compared to the control group. An eight-fold decrease in the APP expression was noted in BM3NP treated group in comparison with the drug control. Notably, the same scenario prevailed in fractalkine expression and BM3NP showed a significant decrease of 1.5-fold ($p < 0.05$) in the mRNA expression compared to KA only group (Fig. 6b). BM3NP treatment showed two-fold reduction in the overexpression of HMGB1 mRNA because of KA-induced excitotoxicity. A significant decrease (1.5-fold) of the relative mRNA amount of FOXO3a was evidenced upon treatment with nanoparticles (Fig. 6b) compared to the control levels.

Downregulation of pro-inflammatory cytokines like IL-1β, IL-6 and IL-8 indicates low seizure susceptibility and organ damage. Increased expression of the anti-inflammatory cytokine IL-1Ra demonstrates neuroprotective and anticonvulsant effects. Administration of BM3NP (4 mg/kg, b.w.) reduced the over-expression of pro-inflammatory cytokines, increased anti-inflammatory cytokines and re-established normal expression of cytokines compared to the control group. A ten-fold decrease in the IL6 expression was noted in BM3NP treated group in comparison with the drug control. Interestingly, the same scenario prevailed in IL-1β expression and BM3NP showed a significant decrease of 6.5-fold ($p < 0.05$) in the mRNA expression compared to KA only group (Fig. 6c). After KA treatment, the transcript level of IL-8 was increased five-fold compared to control group. BM3NP treatment showed a five-fold reduction in the overexpression of IL8 mRNA because of KA-induced excitotoxicity. IL-1Ra mRNA expression decreased from 1 to 0.76-fold after the KA treatment. BM3NP treatment resulted in a slight increase (0.76 to 1.15-fold) of the relative mRNA amount of IL-1Ra (Fig. 6c) compared to KA only group.

Administration of BM3NP (4 mg/kg b.w.) reduced the over-expression of pro-inflammatory cytokines (COX-2, LOX, TNFβ, IL-1, IFNγ and MCP-1) and increased anti-inflammatory (IL10) cytokine and re-established normal expression compared to the control group.

3.10. Expression of neuroreceptors

BM3NP treatment decreased the expression of AMPA glutamate receptor subunits - G1, G2, G3 and G4 compared to KA only group. Expression of GluA1(G1) get normalized after 4 mg/kg, b.w. of BM3NP treatment. GluA2(G2) and GluA3(G3) expression decreased 0.5-fold compared to KA control group. Expression of GluA4(G4) decreased 0.2-fold after 4 mg/kg, b.w. of BM3NP treatment.

3.11. Expression of mTORC1 upstream regulators and downstream effectors

Dysregulation of mTORC1 signaling has been linked to impaired synaptic plasticity and cognitive deficits observed in neurological disorders. BM3NP, administered at a dose of 4 mg/kg, b.w., resulted in reduced expression of PI3K, MAPK, BDNF, 4E-BP1, p70S6K, and S6 compared to the KA control group. mRNA expression of PI3K, MAPK and BDNF is decreased by 0.2, 0.4 and 0.2-fold, respectively, compared to KA only group. Similarly, mRNA transcript levels of 4E-BP1, p70S6K and ribosomal protein S6 are reduced 0.6, 0.2 and 1-fold, respectively.

3.12. Western blot analysis

A western blot analysis was conducted specifically to investigate any alterations in the protein expression levels of the AMPA glutamate receptor subunits after administration of BM3 (4 mg/kg, b.w.), BM3NP (4 mg/kg, b.w.) and LEV (4 mg/kg, b.w.) along with KA (10 mg/kg, b.w.). Expression of both non-phosphorylated and phosphorylated forms of AMPA glutamate receptor subunits protein was detected at around 100 kDa (Fig. 7a). Expression of Glu-A1 AMPA is increased after the treatment of BM3, but the administration of its nanoencapsulated form (BM3NP) decrease the overexpression and generated a beneficial environment for the tissues. Expression of phosphorylated-Glu A1 AMPA protein (at Ser-845) itself did not show any obvious change after BM3/BM3NP treatment compared to control group. In addition, no seizure-induced changes in the expression of Glu-A2 AMPA glutamate receptor subunits were observed after BM3/BM3NP treatment compared to the control group. Phosphorylation of GluA2 (at Tyr869/Tyr873/Tyr876) increased significantly following BM3 injection compared with the control group. Moreover, BM3NP treatment decreases the over-expression of phosphorylated Glu-A2. It was found that BM3NP treatment decreased the expression level of Glu A3 AMPA protein even below the basal level. Protein expression level of Glu A4 AMPA subunits remained unaltered in the brain tissues after BM3NP treatment compared to the control and drug control groups. Overall, BM3NP treatment was significantly more effective than LEV, in inhibiting the seizure-induced changes in the protein expression of AMPA glutamate receptor subunits. Furthermore, no change in the expression of β-actin was observed in any of the treated groups.

Effect of BM3/BM3NP treatment on seizure activation in the brain tissues was investigated by monitoring the protein expression of phospho-mTOR, mTOR, P70s6kinase (Thr), P70s6kinase (Ser) and phospho-4EBP1. Treatment groups were intravenously injected with BM3 (4 mg/kg, b.w.), BM3NP (4 mg/kg, b.w.) and LEV (4 mg/kg, b.w.) in each group along with intraperitoneal administration of KA (10 mg/kg, b.w.). Results suggest that phosphorylation of mTOR at Ser-2448 does not increase and remained unaffected after BM3/BM3NP treatment compared to the control group. In addition, mTORC1 also phosphorylates and inhibits the activity of the eukaryotic initiation factor 4E (eIF4E) - binding protein 1 (4E-BP1), leading to its dissociation and initiation of translation of proteins by associating with other initiation factors. Expression of phosphorylated p70S6K (Thr389), a downstream target of mTORC1, was slightly increased than normal level. Results revealed an overexpression in the levels of p70S6K (Ser371), in the brain compared with control animals. Expression of phospho-4EBP1 did not show any change in any of the treated groups. β-actin expression remained unaltered in the brain tissues of all treated groups.

4. Discussion

Nanoparticles (1–1000 nm) facilitate drug delivery across the blood–brain barrier by binding, entrapping, or transporting drugs via transcytosis or endocytosis (Saraiva et al. 2016). PLGA nanoparticles are efficient drug delivery systems capable of crossing the BBB. PLGA has excellent biocompatibility and biodegradability. After drug delivery, it is hydrolyzed into lactic and glycolic acids, which are naturally-occurring metabolites. PEGylation contributes to enhanced aqueous solubility, stability, low intermolecular aggregation, minimum immunogenicity, and improved blood circulation time (Sekhar et al. 2019).

Zeta potential is a vital tool for the anticipation of the stability of nanoparticles. Most of the NP formulations for brain delivery have negative zeta potentials (between − 1 to − 45 mV) which enables drug penetration across biological membranes (Kunjumon et al. 2021b; Sekhar et al. 2021). Likewise, the present study illustrated that BM3NP have an ideal size of 165.5 nm with an anionic zeta potential (-32.5 mV) and a suitable PDI value of 0.04. Results suggest that these nanoparticles are suitable for drug delivery applications. BM3 molecules were released in a controlled manner from the copolymer, with 50% released after 15 hours in phosphate buffer at physiological pH (Sekhar et al. 2025).

The SEM and TEM analyses found that spherical BM3NP nanoparticles showed uniform distribution. The nanoparticles also reduced damage caused by KA and restored the brain's ultrastructure, including normal myelinated neurons (Kunjumon et al. 2021b).

Present study found no significant changes in physical, biochemical, or hematological parameters or body weight in BM3/BM3NP treated animals compared to the control group over 14 days. Histopathological analysis demonstrated that there were no indications of toxicity observed in any organs, when compared to the control group.

The study assessed the potential of BM3/BM3NP treatment in reducing oxidative damage induced by KA. It found that brain tissues were effectively protected from oxidative stress, with improved trolox equivalent levels and reduced MDA levels. BM3NP treatment also increased GPx, SOD, and GR activity, highlighting the beneficial effects of antioxidant enzymes (Padurariu et al. 2010; Tandogan et al. 2011).

BM3NP treatment effectively reduced seizure activity induced by KA, as observed in the Racine scale scores displayed in Fig. 5. BM3NP administration protected against KA-induced brain membrane permeability. These findings align with previous research on the protective effects of simvastatin, edaravone, and dexamethasone against KA-induced changes in a co-culture BBB model (Barna et al. 2020).

AMPA receptors are abundantly expressed in the postsynaptic neuronal membrane and are often increased in epilepsy. Enhanced expression leads to cortical hyperexcitability. (Ying et al. 1998; Anggono and Huganir 2012). By modulating the activity of these receptors, it may be possible to reduce the excitability of neurons and thereby prevent the occurrence of seizures (Song and Huganir 2002). Results

showed that BM3NP decreased Glu-A1 AMPA overexpression and created a beneficial environment for tissues. In addition, BM3NP treatment decreased Glu A3 AMPA protein expression even below the basal level. Glu A4 AMPA subunits remained unaltered.

Seizures from KA activate mTORC1 signaling, controlling protein synthesis and inhibiting 4E-BP1. This pathway is regulated by factors like glutamate receptors, BDNF, ERK, and PI3K/Akt, and is influenced by factors like p70S6K and ribosomal S6 protein kinase (Talos et al. 2012). Results show that the phosphorylation of mTOR at Ser-2448 remained unchanged after the administration of BM3/BM3NP compared to the control group.

Glutamate receptor activation influences mTOR pathway, suggesting a possible relationship between neuronal hyperexcitation, abnormal mTOR activity, and the development of spontaneous seizures. p70S6 kinase is the major protein kinase that phosphorylates mTOR at Ser-2448 in mammalian cells, and its activity is regulated by extracellular amino acids and polypeptide growth factors. Cellular amino acid availability regulates p70S6 kinase activity *via* the TSC/Rheb pathway and causes feedback phosphorylation of mTOR at Ser-2448 (Chiang and Abraham 2005).

BM3NP is proposed to counteract the harmful effects of KA-induced excitotoxicity, which include seizures, changes in behavior, oxidative stress, activation of glial cells, generation of inflammatory mediators, and specific degeneration of neurons in the brain. Prolonged injection of KA can lead to the rupture of brain membranes, the production of ROS, the enhancement of long-term potentiation, the increased excitability of neurons, and the occurrence of seizures. BM3NP treatment before KA treatment protected from apoptosis and reversed the MMP loss. BM3NP treatment safeguards against brain membrane leakage, boosts antioxidant levels, facilitates regular synaptic transmission, and modulates mTOR signaling (Fig. 8).

5. Conclusion

The present study found that BM3NP nanoparticles have an ideal size range, charge, and polydispersity index and are more biocompatible than BM3, making them suitable for drug delivery applications. BM3NP administration (4 mg/kg, b.w.) decreased oxidative stress parameters and exhibited antioxidant properties. BM3NP treatment effectively reduced seizure scores and provided brain membrane integrity. Results showed that BM3NP decreased the overexpression of AMPA receptor subunits, seizure markers like fractalkine, and other pro-inflammatory cytokines. Dysregulated expression of subunits of the mTOR Complex 1 (mTORC1) pathway was normalized after BM3NP administration compared to the control group. Our results suggest that BM3NP could be a potential candidate for seizure control and neuroprotection.

Limitations

This is an acute excitotoxicity model; chronic epilepsy, long-term behavioral recovery, and pharmacokinetics were not evaluated.

Abbreviations

BM3- Bacoside A; BM3NP- Bacoside A nanoparticle; DLS- Dynamic Light Scattering; PLGA-b-PEG- poly (lactic-co-glycolic acid)-block-poly(ethylene glycol); KA- kainic acid; AEDs- antiepileptic drugs; AMPA- α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; APP- amyloid beta protein precursor; HMGB1- high mobility group box 1; mTORC1-mammalian target of rapamycin complex 1; MAPK- mitogen-activated protein kinases; BDNF- brain-derived neurotrophic factor; NO- nitric oxide; EE%- percentage of encapsulation efficiency; BBB- blood brain barrier.

Declarations

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Conflict of Interest

The authors declare no competing interests.

Author contributions: CRediT

Vini C. Sekhar: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Sabulal Baby:** Project administration, Conceptualization, Funding acquisition, Writing - review & editing. **Gayathri Viswanathan:** Conceptualization, Methodology, Formal analysis, Project administration, Funding acquisition, Writing - original draft, Writing - review & editing.

Data availability

The datasets used and/or analyses conducted in this study are available from the corresponding author upon reasonable request.

Ethics approval and Consent to Participate

The Institutional Animal Ethics Committee (IAEC) of Jawaharlal Nehru Tropical Botanic Garden and Research Institute approved all experimental procedures (No. B1/05/2016/PPD-02), which were

conducted following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Consent of Publication

Not applicable.

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Figures

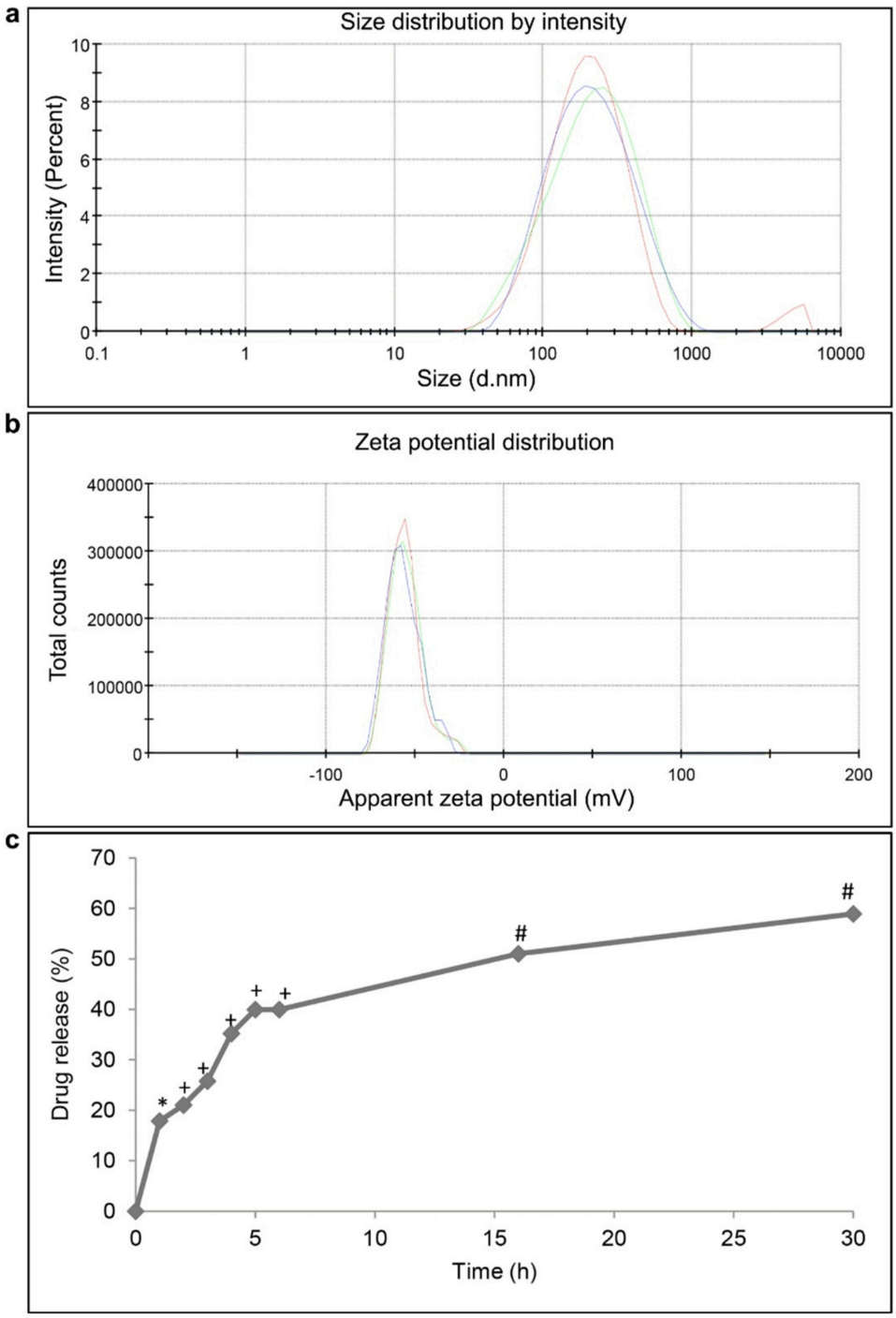


Figure 1

Physicochemical characterization of the BM3NP **a**) DLS, **b**) zeta potential, **c**) release kinetics. Independent sample student's t-test, *$p < 0.05$, $^{+}p < 0.01$, $^{\#}p < 0.001$ compared to the initial drug release.

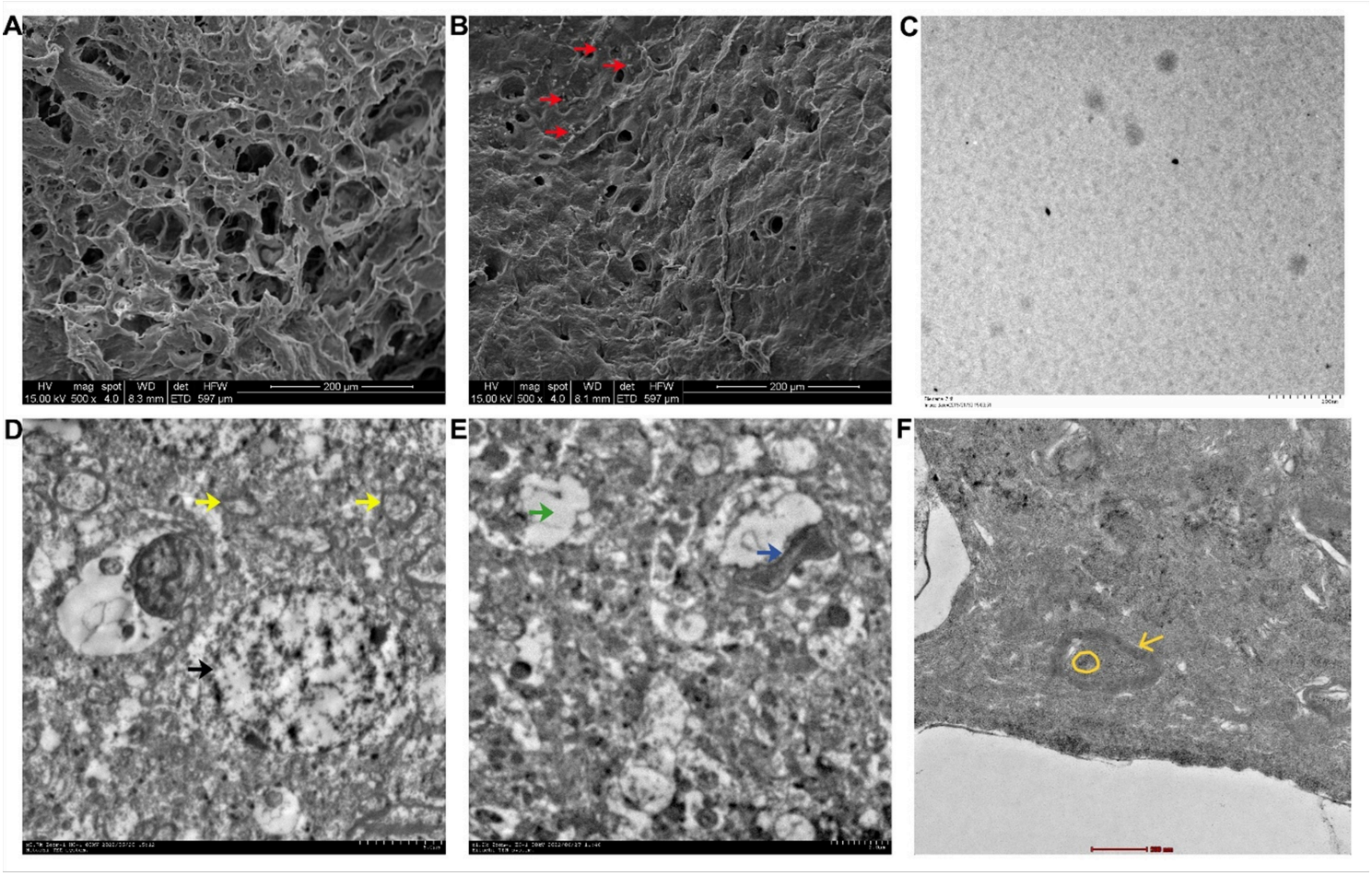


Figure 2

Ultrastructural changes of mice brain and BM3NP distribution using scanning electron microscopy. SEM image of (A) control and (B) BM3NP+KA-treated mouse brain; red arrows indicate spherical nanoparticles. (C) Chemical TEM image of BM3NP depicting the spherical character of nanoparticles (D, E, F) TEM images of the brain tissues; (D) brain tissue images of control group with regular nuclear outline (black arrow) and myelinated neurons (yellow arrows), (E) KA-induced sample showing distorted endothelial cells (blue arrow), cytoplasmic swelling and severe neural oedema (green arrow), (F) brain tissue images of BM3NP (4 mg/kg, b.w., i.v.)+KA group with regular myelinated neurons (yellow arrow) and intracellular localization of BM3NP (within yellow circle) as electron-dense particles.

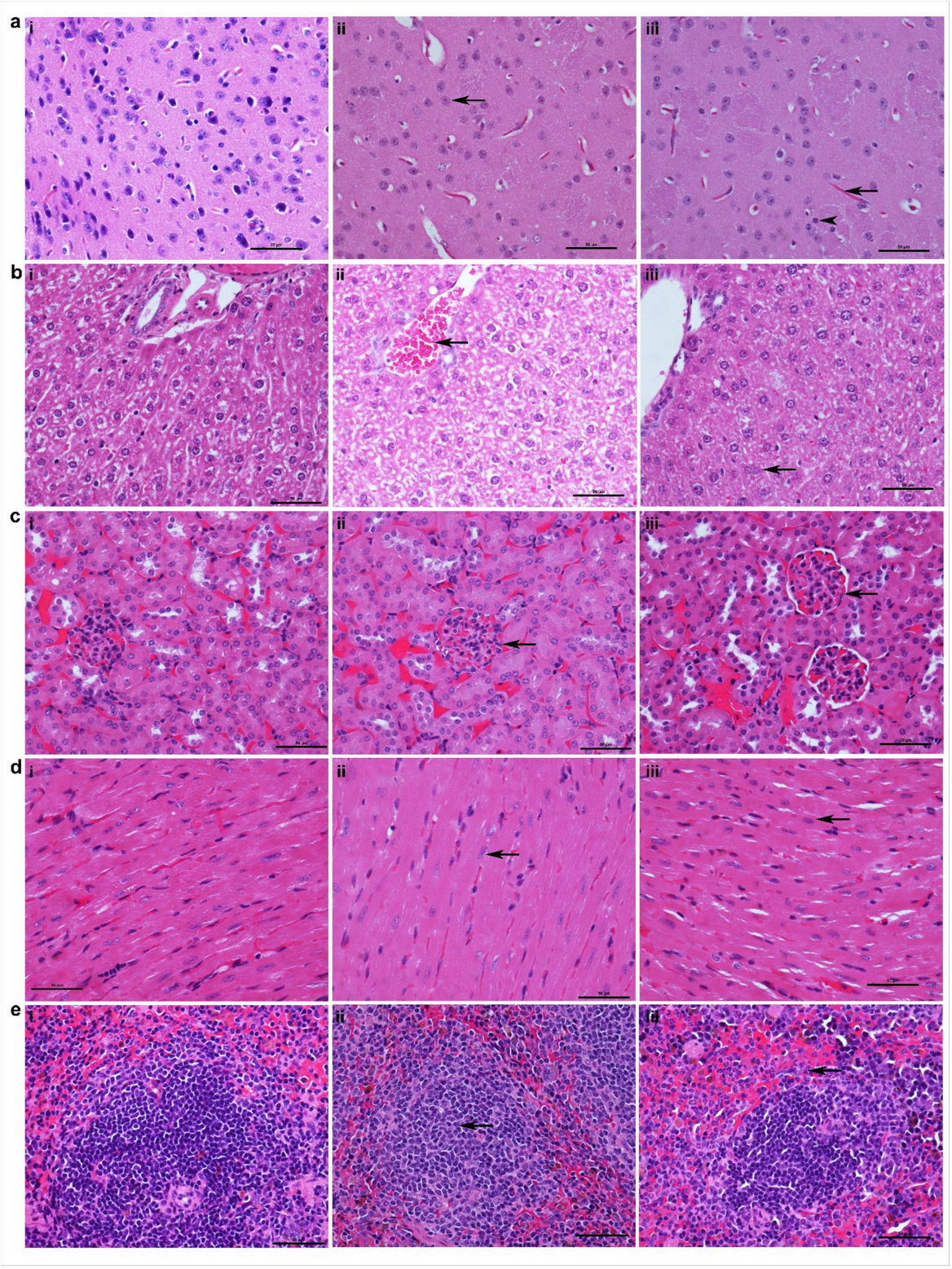


Figure 3

a) Light microscopic images of the brain sections from different treatment groups. (i) Control mouse brain section showing normal brain architecture; (ii) BM3 treated group- normal cerebral cortex, neuronal cell bodies with prominent round nuclei (arrow) (iii) BM3NP treated group- no abnormality could be detected with normal cerebral cortex; glial cells (arrowhead); capillary (arrow) **b)** Light microscopic images of the liver sections from different treatment groups, (i) control mouse liver section showing

normal liver architecture, (ii) BM3 treated group-healthy hepatocytes with some signs of regeneration indicated by bi-nucleated cells, hepatic sinusoids having red blood cells (arrow) (iii) BM3NP treated group- healthy hepatocytes with bi-nucleated cells (arrow) and few MNCs observed in the sinusoids; **c)** Light micrographs of the kidney sections from the different treatment groups, (i) control kidney section showing normal renal cortex and glomerular tufts, (ii) BM3 treated group- healthy glomeruli (arrow) and proximal and distal convoluted tubules, (iii) BM3NP treated group- healthy glomeruli (arrow) and collecting tubules appeared without any abnormality; **d)** Light micrographs depicting the changes in the heart sections from the different treatment groups, (i) control group- there were no anomalies in the heart muscle cells, (ii) BM3 treated group- healthy cardiac myocytes (arrow); no abnormality could be detected in myocardial fibers, (iii) BM3NP treated group- healthy cardiac myocytes (arrow); no degeneration and necrosis were observed in the cardiac tissue; **e)** Light microscopic images of the spleen sections from the different treatment groups, (i) control spleen section showing normal splenic architecture with normal lymphoid follicles and sinuses, (ii) BM3 treated group- normal lymphoid tissue with active proliferation in the white pulp region (arrow), (iii) BM3NP treated group- well-defined spleen section with normal capsule and lymphoid tissue (arrow).

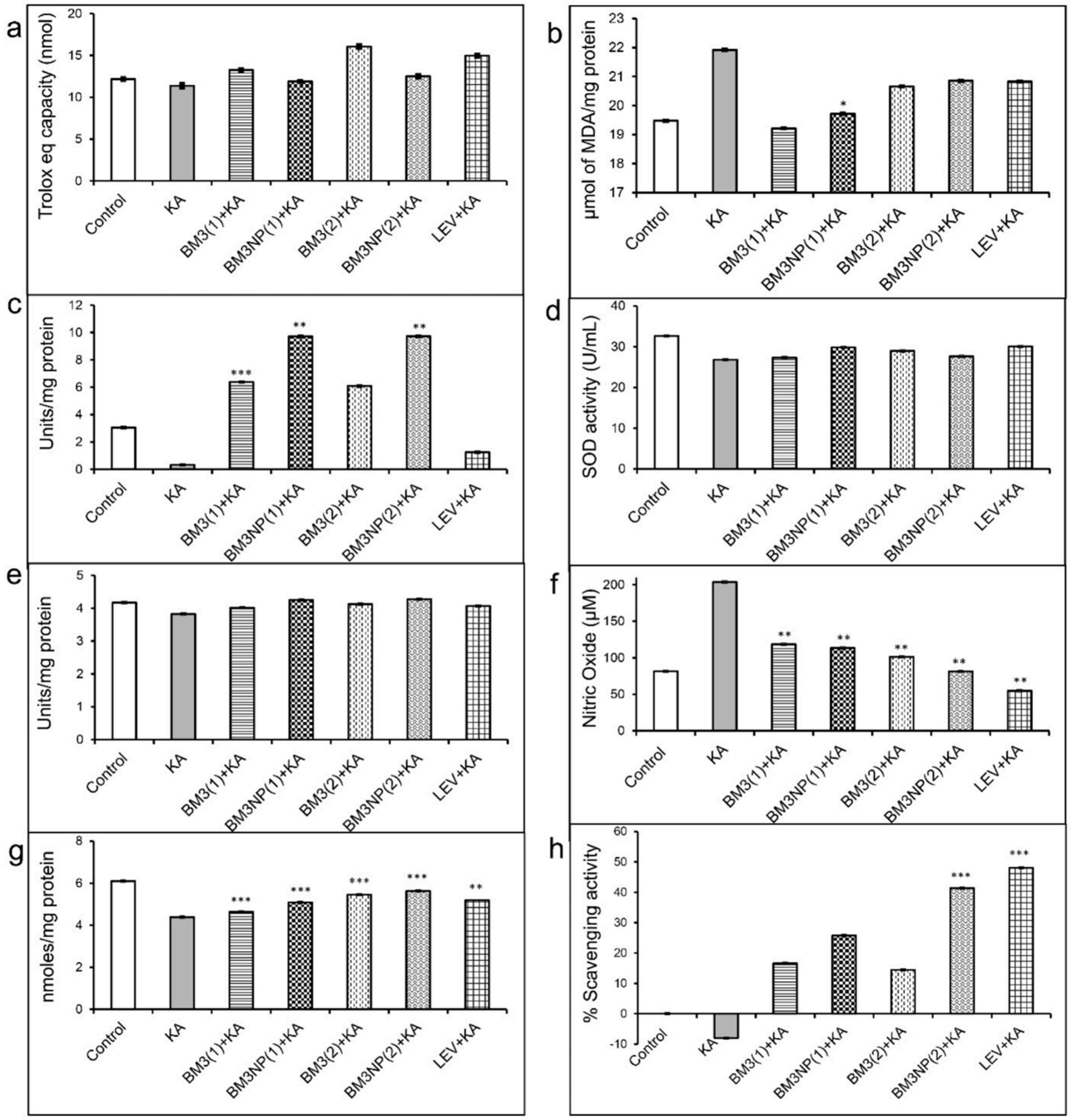


Figure 4

a) Trolox equivalent capacity measured in mouse brain tissue lysates, showing quantity (nmol) per mg of extracted protein **b)** Level of malondialdehyde in the brain tissues of mice exposed to BM3/BM3NP **c)** Glutathione peroxidase activity in the mice brain of control and treated groups **d)** Superoxide dismutase activity in the mice brain of control and treated groups **e)** Glutathione reductase activity in the mice brain of control and treated groups **f)** Nitric oxide concentration in the mice brain of control and treated groups **g)** GSH level in the mice brain of control and treated groups **h)** Percentage of H_2O_2 scavenging activity in

the mice brain of control and treated groups. BM3(1)+KA and BM3NP(1)+KA represent treatment with 4 mg/kg, b.w. of BM3 and BM3NP, respectively. BM3(2)+KA and BM3NP(2)+KA indicate treatment with 8 mg/kg, b.w. of BM3 and BM3NP, respectively. LEV + KA denotes the treatment with levetiracetam (4mg/kg, b.w). Values are expressed as mean ± SD, n = 6. Independent sample student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001 compared to the KA only group.

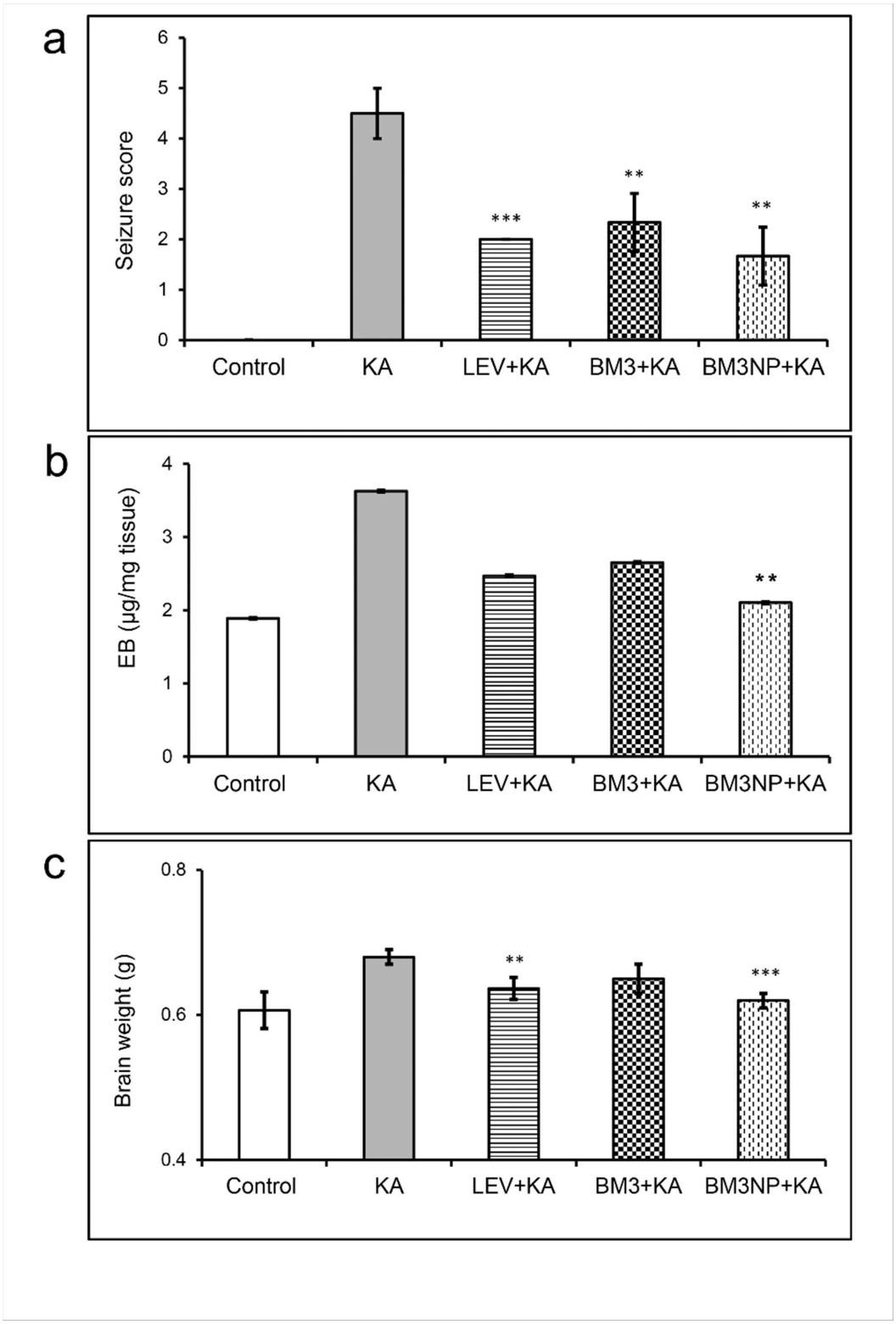


Figure 5

a) Racine convulsion scale scores of mice exposed to 4 mg/kg, b.w. of BM3/BM3NP. **b)** Evaluation of brain membrane integrity of mice using Evan's blue. **c)** Determination of brain weight of mice after BM3/BM3NP+KA treatment (values are mean ± S.D., n = 6). Independent sample student's t-test, **p < 0.01, ***p < 0.001 compared to the KA only group.

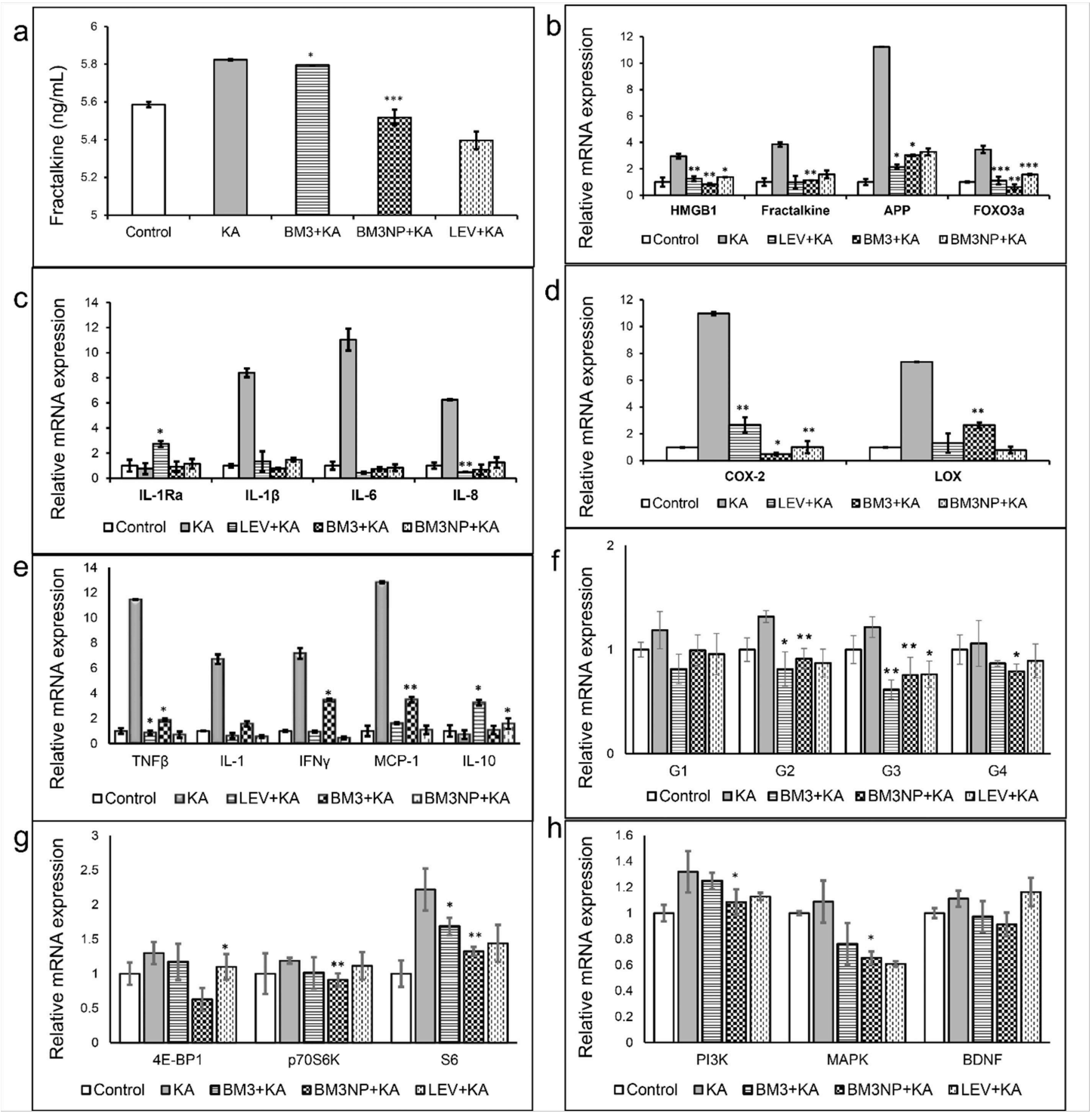


Figure 6

a) Fractalkine protein expression against KA induced excitotoxicity in mice exposed to 4 mg/kg, b.w. of BM3/BM3NP. **b)** mRNA expression of seizure markers- HMBG1, fractalkine, APP and FOXO3a against KA induced excitotoxicity in mice brain exposed to 4 mg/kg, b.w. of BM3/BM3NP. **c)** mRNA expression of pro- and anti-inflammatory cytokines- IL-1Ra, IL-1β, IL-6 and IL-8 against KA induced excitotoxicity in mice brain exposed to 4 mg/kg, b.w. of BM3/BM3NP. **d)** mRNA expression of COX-2 and LOX against KA induced excitotoxicity in mice brain exposed to 4 mg/kg, b.w. of BM3/BM3NP. **e)** mRNA expression of TNFβ, IL-1, IFNγ, MCP-1 and IL-10 against KA induced excitotoxicity in mice brain exposed to 4 mg/kg, b.w. of BM3/BM3NP. **f)** AMPA glutamate receptor subunits - G1, G2, G3, G4 expression after administration of BM3 (4 mg/kg, b.w.) and BM3NP (4 mg/kg, b.w.) along with KA (10 mg/kg, b.w.) and LEV + KA (levetiracetam, 4 mg/kg, b.w.). **g)** mRNA expression of downstream effectors of mTORC1 signaling- 4E-BP1, p70S6K and ribosomal protein S6 after administration of BM3 (4mg/kg, b.w.) and BM3NP (4 mg/kg, b.w.) along with KA (10 mg/kg, b.w.) and LEV + KA (levetiracetam, 4 mg/kg, b.w.). **h)** mRNA expression of upstream activators of mTORC1 signaling- PI3K, MAPK and BDNF after administration of BM3 (4 mg/kg, b.w.) and BM3NP (4 mg/kg, b.w.) along with KA (10 mg/kg, b.w.) and LEV + KA (levetiracetam, 4 mg/kg, b.w.). Values are reported as mean ± S.D., with n = 6. An independent sample Student's t-test was conducted, with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by comparing with the KA only group.

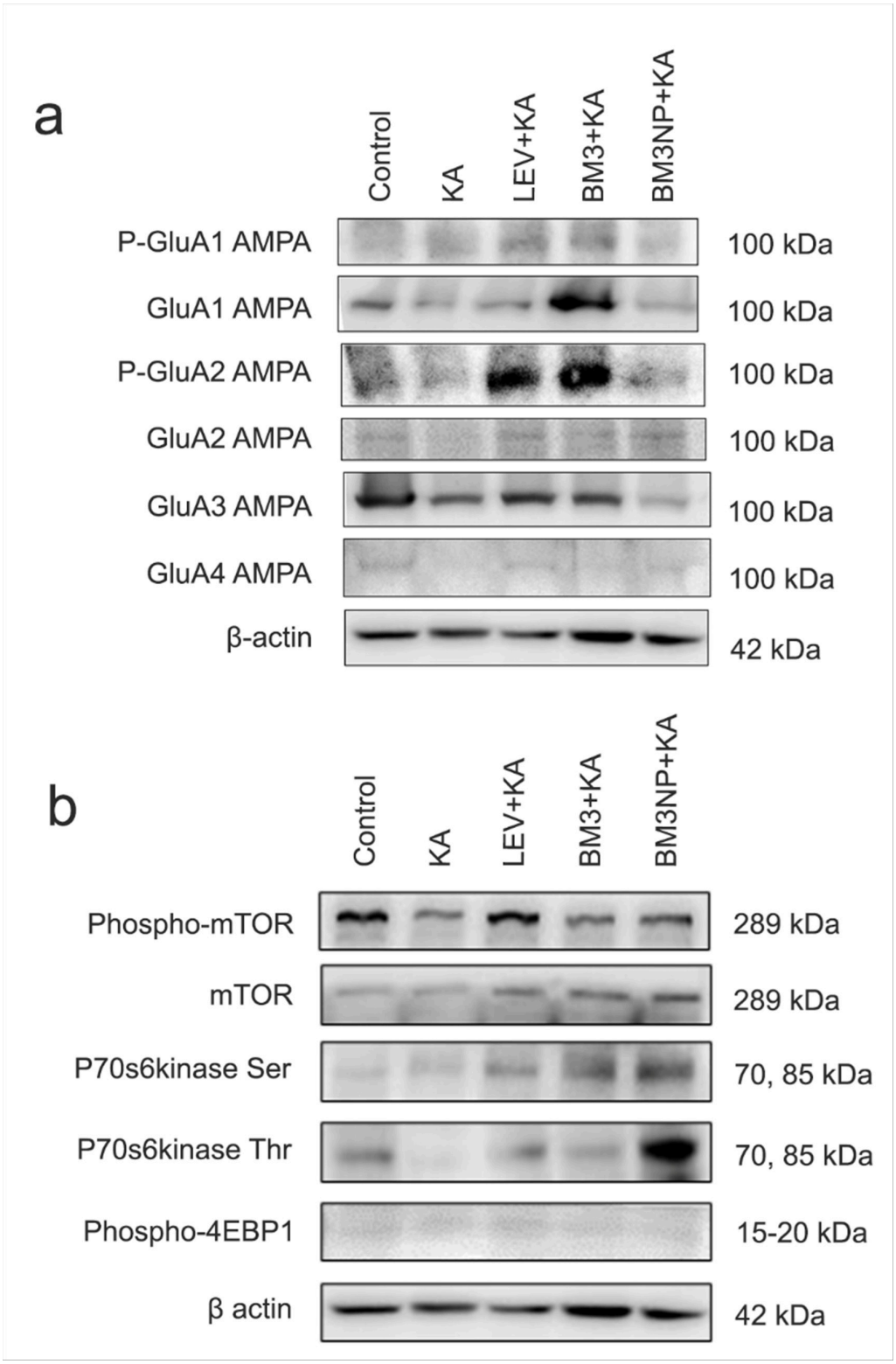


Figure 7

a) Protein expression of GluA1-A4 AMPA receptors, phospho-GluA1 AMPA and phospho-GluA2 AMPA after administration of BM3/BM3NP/LEV (4 mg/kg, b.w.) + KA (10 mg/kg, b.w.). **b)**Protein expression of phospho-mTOR, mTOR, P70s6kinase(Thr), P70s6kinase(Ser) and phospho-4EBP1 after administration of BM3/BM3NP/LEV(4 mg/kg, b.w.) + KA (10 mg/kg, b.w.).

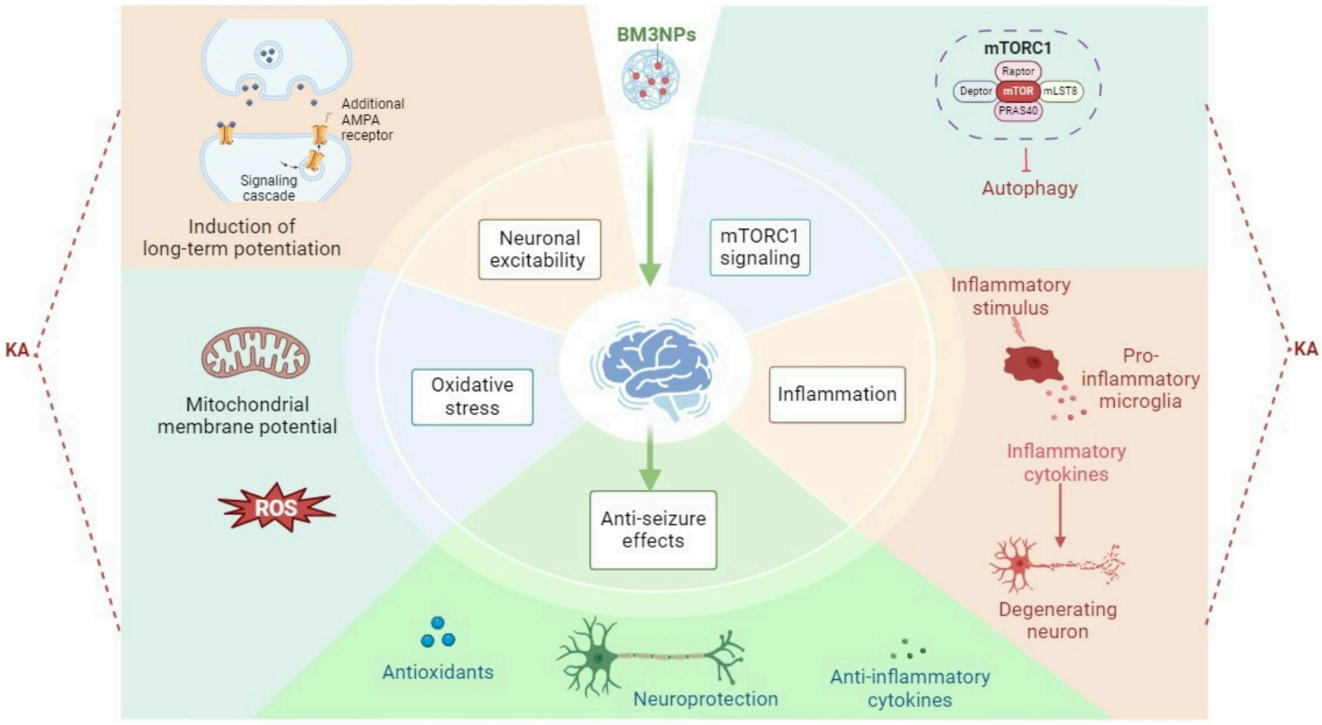


Figure 8

KA-induced excitotoxicity causes oxidative stress, over-expression of inflammatory cytokines, neurotoxicity, and neurodegeneration. It induces abnormal neuronal excitability and causes long-term potentiation. It leads to dysregulation of mTOR signaling and inhibition of autophagy. BM3NPs promote neuroprotection by increasing antioxidant levels, upregulating anti-inflammatory cytokines, promoting normal synaptic transmission and regulating mTOR signaling.