Rivastigmine Loaded PEG-PLGA Nanoparticles for Enhanced Delivery to the Brain: In-Vitro and In-Vivo Studies for Alzheimer's disease

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Abstract

Purpose

Rivastigmine Tartrate (RT) is a reversible cholinesterase inhibitor used for the treatment of Alzheimer's disease and CNS disorder. The entry of the drug to the brain is restricted is due to the presence of the Blood-Brain Barrier (BBB). In this work, we have developed amphiphilic copolymer, poly (ethylene glycol)-poly (lactide-coglycolide (PEG-PLGA) loaded RT nanoparticles to enhance brain delivery.

Methods

Rivastigmine-loaded PEG-PLGA nanoparticles were prepared using the nanoprecipitation technique. Pegylated polyethylene glycol (polymer) and Pluronic F68 (Surfactant) were selected as preparation of Polymeric Nanoparticle. Characterization studies on the formulation such as DSC, Zeta potential, SEM, and Drug Entrapment were conducted. In-vitro drug release, Pharmacokinetic, and Pharmacodynamics studies were performed.

Results

The prepared nanoparticles are spherical and the size ranges from a minimum of 125.93 ± 0.55 to a maximum of 179.60 ± 1.06 nm. In vitro release studies in PBS pH 7.4 showed a biphasic release pattern of the drug from the nanoparticles. Pharmacokinetic studies in male Wistar rats showed an increase in the C_{max} of RT when administered as PEG- PLGA nanoparticles than RT administered as a solution in PBS and a notable 3.5fold increase in the $T_{1/2}$ also was observed. Tissue distribution studies also showed a 4fold increase in the amount of RT as nanoparticles that have entered the brain when compared to RT in PBS.

Conclusion

The Smaller size of the nanoparticles along with the presence of hydrophilic PEG group has assisted in enhancing the concentration of Rivastigmine in the brain which has consequently even improved the memory learning skills of the animals

1. Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder associated with the social and behavioral skills of people. It leads to an irreversible, progressive brain disease that slowly destroys memory and thinking skills. The characteristic feature of the disease includes an accumulation of amyloid plaques and neurofibrillary tangles along with chronic inflammation in the neurons [7]. The basic reason for the

development of cognitive dysfunction is reported as selective attenuation acetylcholine-based transmission of signals in the neuronal network.

The current treatment strategies approved by the USFDA include the usage of drugs like rivastigmine, donepezil, and galantamine which enhances acetylcholine (a neurotransmitter associated with thought, memory, and other cognitive processes levels in the brain. But the main problem posed by those drugs is their inability to reach the brain in higher concentrations owing to the presence of the blood-brain barrier. In the future even if several newer drugs are discovered they may fail in treating AD because of the blood-brain barrier which restricts the entry of several drugs and molecules to the brain [1]. Hence, there is a necessity to develop a delivery system that can efficiently deliver the drug to the brain.

Nanotechnology has opened doors for the design and development of smarter drug delivery systems below 200 nm that can efficiently enhance the delivery of molecules to the brain. In this research work, an attempt was made to develop a delivery system for Rivastigmine tartrate using polyethylene glycol-polylactide- co-glycoside as a polymer. PEG PLGA is an amphiphilic polymer consisting of the lipophilic poly (D, L-lactic-co-glycolic acid) (PLGA) moiety attached to the hydrophilic PEG portion [6], [10]. PLGA is a biodegradable and non-toxic polymer with good controlled release properties. Long-circulating and stealth nanoparticles can be obtained by chemical modification of polymers with certain hydrophilic synthetic polymers such as poly (ethylene glycol) (PEG). These polymers prevent opsonization owing to their neutral charge and hydrophilicity. Further, the hydrophilic nature of the polymer surface promotes permeation across the BBB [15, 16], [4, 5].

Rivastigmine tartrate is a reversible cholinesterase inhibitor used in the treatment of Alzheimer's disease which can't cross the BBB. Despite several attempts made by researchers to prepare nanoparticles for RT, there have been shortfalls in terms of either preparation methods or opsonization of the particles by the macrophages or stability issues or lack of promising response of the animals towards the nanoparticles [1],[15]. In this research work, RT-loaded nanoparticles of PEG PLGA nanoparticles (RT PEG-PLGA) were prepared to check for the ability of the PEG surface to enhance the uptake in the brain. The prepared nanoparticles were evaluated for their size, shape, zeta potential, and physical form of the entrapped drug. Further, *in vivo* pharmacokinetic and pharmacodynamic studies were performed in a suitable animal model to evaluate the ability of the potential of nanoparticles to reach the brain and to check its potency in rats induced with amnesia.

2. Materials And Methods

2.1 Materials and reagents

Materials

Rivastigmine tartrate (RT) was obtained as a gift sample from Ranbaxy laboratories Pvt. ltd, Delhi. Polyethylene glycol (PEG average molecular weight 2000- Poly (D, L-lactide - Co-Glycoside) (PLGA molecular weight 5000), Dichloromethane (DCM), and Scopolamine was purchased from Sigma –Aldrich

India. Pluronic F68 was purchased from Himedia laboratories. Orthophosphoric acid, Sodium Hydroxide, Acetone, and Phosphate buffer used in the study were collected from Vels University Central Chemical stores.

2.2 Preparation of Rivastigmine loaded PEG - PLGA Nanoparticles

RT loaded PEG-PLGA (RT PEG- PLGA) nanoparticles were prepared by the nanoprecipitation technique [2]. Briefly, PEG-PLGA 50 mg and rivastigmine 10 mg were dissolved in dichloromethane and added to 10 ml of an aqueous solution containing Pluronic F68. This mixture was emulsified by ultra-sonication (PRO SC 250) for 10 minutes. The organic solvent was then allowed to evaporate by stirring at 500 rpm for 4 hours on a magnetic stirrer. Various batches of formulation were prepared by varying the amount of polymer, the volume of the organic phase, and concentration of surfactants as shown in Table 1.

Particle size

The particle size of the formulations was evaluated using Malvern Zetasizer (Malvern Nano ZS 90, UK). Briefly, a sufficient amount of the formulation was filled in a disposable polystyrene cuvette and analyzed for the particle size. Each experimental trial was carried out in triplicate [12].

Zeta potential

The zeta potential of the formulations was estimated using Malvern Zetasizer (Malvern Nano ZS 90, UK). The sample was filled in disposable polystyrene cuvette and the zeta potential of the sample was analyzed using a zeta dip cell.

Scanning Electron microscopy (SEM)

The surface morphology of the particles was studied using Scanning Electron Microscopy (Leica Cambridge S 360; Leica Microsystems, Wetzlar, Germany). The liquid sample suspension was placed over the carbon tape, dried overnight, and visualized for the morphology of the nanoparticles [12].

Differential Scanning Calorimetry

The physical state of pure RT, PEG- PLGA polymer, Pluronic F 68, a physical mixture containing RT, PEG-PLGA and Pluronic F68, and RT- PLGS NPS was found using Shimadzu-60. The samples were sealed in aluminum pans and the temperature was increased at a constant rate.

Entrapment efficiency

The entrapment efficiency of the formulations was found by estimating the amount of free drug present in the nanoparticles after ultra-centrifugation. 2 ml of the sample was centrifuged at 20,000 rpm for 15

minutes using an ultracentrifuge. The supernatant solution was separated and the amount of entrapped drug was estimated using the following equations [20].

$$\text{Encapsulation efficiency (\%)} = \frac{\text{amount of drug added during preparation} - \text{amount of free drug in the supernatant} \times 100}{\text{amount of drug added during preparation}}$$

In vitro **drug release studies**

In vitro, drug release studies were performed using the dialysis bag method for RT PEG-PLGA NPs. Briefly, the sample (equivalent to 2 mg of RT) was sealed in dialysis membrane using membrane clips. The sealed membrane was placed in a beaker containing 50 ml of pH 7.4 phosphate buffer saline and kept for stirring at 100 rpm at 37 ± 1 ºC. Periodically 1 ml of the medium was withdrawn at pre-fixed time intervals and replaced with fresh buffer to maintain sink conditions. The amount of drug present in the withdrawn samples was estimated using UV spectroscopy at 219 nm [19].

Bio-analytical method development

The amount of RT present in the biological samples was analyzed using HPLC (Shimadzu LC2010) with a UV detector using a C_{18} column (Inertsil, 220 X 4.6mm) maintained at 25ºC). The flow rate of the mobile phase (a mixture containing 80:20 parts of 20 mM phosphate buffer and acetonitrile adjusted to pH 3.1 using orthophosphoric acid) was maintained at 0.3 ml/min. During the analysis, 20 µl of the samples were injected and the samples were detected at 219 nm. The method was validated in terms of specificity, linearity, and reproducibility. The r^2 value was found to be 0.998, and the retention time for RT was found to be at 0.88 min.

In vivo **pharmacokinetic studies**

In vivo pharmacokinetic studies were carried out in male Wistar rats weighing about 200-250g [1]. The protocol for the animal experiments was reviewed and approved by Animal Ethical Committee at Vels University, Chennai. The IAEC No: XVIII/VELS/PCOL/11/2000/CPCSEA/IAEC/04.02.2016. The animals were kept in the animal house at 20 ± 2º C and 50–60% RH. The animal house was well ventilated and the animals were provided with food and water ad libitum and maintained on a standard diurnal cycle in a cage with sterile paddy husk. The animals were allowed to fast overnight before the study was performed. Three groups of rats each containing 6 numbers in each group were used for pharmacokinetic study. A dose equivalent to 1 mg/kg was administered by intravenous (i.v) route through the tail vein after suspending in phosphate buffer saline. Group 1 received RT solution dissolved in PBS 7.4 (RT PBS), Group 2 received RT PEG-PLGA nanoparticles and Group 3 served as control. Blood samples were periodically collected by retro-orbital venous plexus puncture with the aid of a capillary tube at 0.25, 1, 2, 4, 8, 12, and 24 hours post-dosing in microcentrifuge tubes containing 10 µl of sodium citrate. The blood

samples were centrifuged at 4000 rpm for 10 min and the plasma was separated and stored at -20ºC until analysis by high-performance liquid chromatography. The pharmacokinetic parameters were calculated through a model-independent or non-compartmental model using Kinetica software (Adept Scientific, United Kingdom).

Brain distribution studies

In vivo brain distribution studies were carried out in male Wistar rats weighing about 200-250g [8]. Three groups of rats each containing 6 numbers in each group were administered with a dose equivalent to 1mg/kg by intravenous (i.v) route through the tail vein. Group 1 received RT solution dissolved in PBS 7.4 (RT PBS), Group 2 received RT-loaded nanoparticles (PEG-PLGA NPS) and Group 3 served as control. After 1 hour and 4th -hour post-administration, 3 animals from each group were sacrificed and the target tissue of interest, the brain was removed and homogenized using a tissue homogenizer (Remi) with ice-cold PBS 7.4. The amount of drug in the tissue homogenates was identified using high-performance liquid chromatography.

Extraction of RT from biological samples

RT was separated from the plasma and tissue samples using a modified liquid-liquid extraction method [11]. Rat plasma or tissue homogenate (100µl) was mixed with 20µl of 1M sodium hydroxide solution and vortexed for 120 seconds. 1 ml of tetra butyl methyl ether was added to this mixture and centrifuged at 10, 000 rpm for 15 minutes. The organic layer was separated and evaporated using a turbo vap LV (Biotage USA) under a nitrogen gas stream. This dried residue was reconstituted with 100 µl of a mixture containing 80:20 parts of 20 mM phosphate buffer and acetonitrile adjusted to pH 3.1 using orthophosphoric acid was used as the mobile phase.

3. Pharmacodynamic Studies

Establishment of in vivo model

To evaluate the ability of RT-loaded PEG- PLGA nanoparticles to enhance neurocognition and memory, amnesia was induced in rats using scopolamine which impairs the neurocognitive ability [9]. Neuropharmacological studies were performed in four groups of rats containing 6 numbers in each group. Group 1 was administered with RT PBS, Group 2 was administered with RT PEG-PLGA nanoparticles, Group 3 served as control and Group 4 served as a positive control. The following neuropharmacological studies were performed under pharmacodynamic studies:

Morris' Water Maze Test

The Morris Water Maze (MWM) model has been used extensively to investigate spatial learning and memory in rodents. The experimental apparatus consists of a circular water tank (diameter × height = 100 cm × 35 cm) containing water at 28ºC to a depth of 15 cm, surrounded by powdered milk. After several

trials, the test was conducted on the 5th day after injection of Aβ. During each training trial, the time needed to escape onto the platform was recorded [9].

Elevated Maze Test

The apparatus comprised two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 40 cm) which extend from a common central platform and opposite to each other. The maze is elevated to a height of 50 cm. one hour after the administration of the drug, the animal was placed at the end of the open arms (facing away from the center of the maze) and the time to move from the open arms to the closed arms was noted as transfer latency (TL). The recordings were performed on the first day and at the end of the first day for 90 seconds. TL on the first day will be served as a measure of acquisition learning and TL at the end of 24 hours for retrieval or explicit learning [3].

Estimation of brain acetylcholinesterase

Estimation of acetylcholinesterase (AChE) was conducted in male Wistar rats 24 h after the last pharmacodynamic test [11]. The animals were killed by decapitation and the brains were removed and then homogenized with ice-cold 150 mM KCl. The homogenate was centrifuged at 10,000g for 15 minutes and the supernatant (0.05 ml) was used for estimation of acetylcholinesterase using an assay mixture consisting of 3 ml of 0.01 M sodium phosphate buffer of pH 8, 0.10 ml of Ellman reagent (5,5', dithiobis-(2-nitro benzoic acid)) and 0.10 ml of acetylthiocholine iodide. The levels of AChE were estimated using UV spectroscopy.

Histopathology analysis

Histopathological analysis was carried out in male Wister rats after administration of RT PBS (Group 1) and PEG-PLGA NPs (Group 2). A control group (Group 3) and a positive control group were also selected for the study. Animals were sacrificed after 24 hours and the brains were removed. Brain tissue sections of thickness 7μm were fixed in 10% formaldehyde solution and embedded in paraffin for microscopy study [17]. Finally, the tissues were dipped in water and stained with hematoxylin and eosin for 10 min at 60 ºC. The stained sections were washed in running water to remove excess stain and were then upgraded for dehydration through different grades of alcohol. Finally, the slides were cleared with xylene and mounted with DPX to make them permanent. The sections were observed for the presence of lipofuscin, vacuolization/spongiosis, deposits, and neuronal degeneration.

4. Results And Discussion

Targeting compounds to the brain efficiently requires good permeation across the rigid blood-brain barrier and to retain in the brain for a longer time. Nanoparticles with a size below 200 nm can efficiently cross the blood-brain barrier and can also deliver the drug to the brain. Further, the presence of a hydrophilic coat on the surface of the nanoparticles can enhance the permeation across the blood-brain barrier. Coating the nanoparticles with polysorbate 80 is a well-established technique and has shown good

promise in the delivery of numerous drugs across the blood-brain barrier. In this research work, an attempt has been made to check the effectiveness of polyethylene glycol surface-modified PLGA nanoparticles to enhance the concentration of RT in the brain. RT-loaded PEG-PLGA nanoparticles were prepared by the nanoprecipitation technique method. Optimization studies were performed to identify the effect of variables like the amount of polymer, the concentration of surfactant, and the volume of the organic phase used during the formulation of the nanoparticles.

Influence of parameters on size

Particle size plays an important role in determining the ability of the molecule to cross the brain barrier where in which, a particle size under 200 nm is necessary for crossing the blood-brain barrier [13]. Table 1 gives details about the size of various formulations. All the formulations were below 200 nm and the particle size of the formulations varied from a maximum of 179.60 ± 1.06nm (F25) to a minimum of 125.93 ± 0.55 (F3). The particle size of the formulations was influenced by the number of excipients used in the formulation, PEG- PLGA, Pluronic F68, and dichloromethane.

As observed from Table 1 when the volume of the organic phase and concentration of surfactant was kept constant, an increase in the concentration of the polymer increased the particle size. This increased particle size can be attributed to the improper dispersion of the polymer due to the increased viscosity of the solution. This increased viscosity will lead to improper separation and breakdown of the polymer. Thus, particle size increases with an increase in polymer concentration. Hence, it would be preferable to use lower amounts (10 mg) of polymer to obtain the least particle size.

Table 1
Rivastigmine loaded PEG-PLGA nanoparticulate formulations

Sl.No	Trial	Polymer	Volume of org. phase	Pluronic F68 (mg)	PS (nm)	PDI	ZP (MW)	DEE(%)
1	F1	10	1	0.5	134.00 ± 0.56	0.169	-11	84
2	F2	10	1	1	131.82 ± 0.82	0.147	-12	72
3	F3	10	1	1.5	125.93 ± 0.55	0.197	-11	69
4	F4	10	2	0.5	143.30 ± 0.62	0.184	-10	79
5	F5	10	2	1	137.17 ± 0.55	0.183	-13	77
6	F6	10	2	1.5	131.47 ± 1.50	0.198	-14	76
7	F7	10	3	0.5	162.37 ± 0.68	0.175	-12	81
8	F8	10	3	1	150.70 ± 1.05	0.217	-14	78
9	F9	10	3	1.5	146.13 ± 0.59	0.203	-15	75
10	F10	30	1	0.5	144.20 ± 0.60	0.169	-11	77
11	F11	30	1	1	141.70 ± 0.85	0.147	-11	75
12	F12	30	1	1.5	132.57 ± 1.10	0.197	-12	71
13	F13	30	2	0.5	151.07 ± 1.10	0.184	-10	80
14	F14	30	2	1	145.37 ± 0.67	0.183	-15	78
15	F15	30	2	1.5	140.73 ± 1.00	0.198	-16	76
16	F16	30	3	0.5	169.27 ± 1.21	0.175	-12	83
17	F17	30	3	1	158.87 ± 0.83	0.217	-13	79

Sl.No	Trial	Polymer	Volume of org. phase	Pluronic F68 (mg)	PS (nm)	PDI	ZP (MW)	DEE(%)
18	F18	30	3	1.5	147.13 ± 0.83	0.203	-14	73
19	F19	50	1	0.5	153.37 ± 0.76	0.169	-11	79
20	F20	50	1	1	143.93 ± 0.49	0.147	-13	76
21	F21	50	1	1.5	141.57 ± 0.87	0.197	-15	70
22	F22	50	2	0.5	160.60 ± 0.89	0.184	-11	81
23	F23	50	2	1	157.70 ± 0.90	0.183	-12	79
24	F24	50	2	1.5	151.70 ± 0.95	0.198	-14	75
25	F25	50	3	0.5	179.60 ± 1.06	0.175	-10	85
26	F26	50	3	1	163.57 ± 1.03	0.217	-12	80
27	F27	50	3	1.5	159.77 ± 1.00	0.203	-15	76

PS-Particle Size, PDI-Poly Dispersibility Index, ZP-Zeta Potential, DEE- Drug Entrapment Efficiency, F1-F27-Formulations.

When variables like the amount of the polymer and Pluronic F68 were kept constant, an increase in the volume of organic solvent also led to an increase in the particle size. When the number of dichloromethane increases, the time is taken for it to evaporate also increases thereby increasing the particle size. Hence, lower amounts of organic phase would facilitate faster evaporation and better formation of nanoparticles.

Also, when the amount of polymer and organic phase was kept constant, increased levels of Pluronic F68 showed a decrease in the particle size. An increase in the Pluronic F68 concentration will reduce the surface tension of the solution leading to a better diffusion of the polymer from the organic solvent to the aqueous phase thereby producing smaller-sized particles.

So, to obtain particles with smaller size, it will be preferable to use higher amounts of Pluronic F68 with the least possible amounts of the organic phase and polymer, and this pattern hold for formulation F3

which has the least particle size of 125.93 ± 0.55 nm.

Zeta potential

The zeta potential of the formulation represents the surface change of the individual particles and it indirectly gives information regarding the ability of the particles to remain stable for a long time and to evade opsonization [14]. Neither change in the amounts of polymer and surfactant and volume of organic phase during the formulation had represented a significant effect on the zeta potential of the nanoparticles. The zeta potentials of the formulations were nearly neutral and varied from – 10.8 ± 0.87 mV to – 16.23 ± 1.06 mV.

The adsorption of the PEG block of the PEG PLGA core and polyethylene oxide (PEO) chain of Pluronic F68 act is the reason for obtaining low zeta potential values, around the neutral region. In general, it is hypothesized that zeta potential values above ± mV 30 mV would produce stable nanoparticle systems. But, Pluronic F68 is a stearic stabilizer that creates a shield by adhering around the surface of the nanoparticles thereby will prevent aggregation among the nanoparticles. Hence, these nanoparticles would remain sterically stable and will also evade phagocytosis by the macrophages *in vivo*.

Entrapment efficiency

RT is a hydrophilic drug and usually, nanoprecipitation method for hydrophilic drugs produces nanoparticles with low entrapment efficiency as the drug escapes from the organic phase to the aqueous. The entrapment efficiencies for the formulations were poor with the highest entrapment efficiency of 85% for formulation F25. A good relation was observed between entrapment efficiency and the excipients. With an increase in the amount of organic solvent and polymer, the entrapment efficiency of the formulations increased which can be attributed to an increase in the volume and space for the drug to get partitioned. Thus, the drug gets more room to enter either the polymer or organic phase leading to an increase in entrapment efficiencies. But, an increase in the amount of stabilizer, Pluronic F68, from 0.5 to 1.5% lead to a decrease in the entrapment efficiencies possibly because of enhanced solubility of RT in the aqueous phase. Thus, to have good entrapment efficiency, it would be better to use higher amounts of organic solvent and PEG- PLGA with lower amounts of stabilizer. Applying this same principle, it can be observed that formulation F25 with higher amounts of organic solvent (dichloromethane = 3 ml) and polymer (PEG- PLGA = 50 mg) and lower amounts of stabilizer (Pluronic F 68 = 0.5%) produced the highest entrapment efficiency values.

Surface morphological properties

The morphology of the nanoparticles observed using SEM as shown in Fig. 1, indicated uniformly distributed spherical shaped particles with a smooth texture. There was no aggregation among the particles due to the presence of PEG coating around the nanoparticle's surface. This PEG coat can be confirmed by the presence of a dark layer around the bright nanoparticles.

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Figure 1. SEM image of RT PEG- PLGA nanoparticles

Compatibility study using DSC

The Thermograms of RT, PEG- PLGA, Pluronic F 68 showed a sharp endothermic peak at 129.31 ºC, 63.92 ºC, 56.55 ºC respectively, corresponding to the melting points of the individual compounds as shown in Fig. 2. The thermogram obtained for the nanoparticles did not show the melting curve of RT which indicates that the drug was well encapsulated in the nanoparticles in a molecular state.

Drug release kinetics

In vitro, drug release studies were carried out for 24 hours using the dialysis bag method in phosphate buffer saline of pH 7.4 as the medium. The cumulative percentage release of RT from RT PEG-PLGA was found to be a maximum of 61.33 ± 3.78% (formulation F3) over 24 hours. The drug release profile in Fig. 3 indicates a biphasic release pattern of the drug from the nanoparticles. Initially, around 20% of the drug was released from the nanoparticles and by the 48th hour, nearly 65% of the drug was released in a sustained manner. The initial burst release may be attributed to the drug present in the aqueous phase and adsorbed on the surface of nanoparticles.

Pharmacokinetic and tissue distribution studies

After intravenous (I.V) administration, the concentration of RT in blood Fig. 4 and brain Fig. 5 over some time was estimated in male Wistar rats after administering RT PBS and RT PEG- PLGA. The pharmacokinetic parameters were calculated by the noncompartmental model using WinNonlin (version 5.1). I.V administration of RT PEG- PLGA sustained the release of RT from the nanoparticles which resulted in enhanced and prolonged residence of RT in the body. The C_{max} of RT after administration of RT PEG-PLGA (1625 ng/ml) was comparatively higher than the C_{max} obtained after administration of RT PBS (1754 ng/ml). An increase in $T_{1/2}$ of RT in plasma from 5.8 to 14.6 h after administration through nanoparticles indicates the sustained release nature of PEG-PLGA and the additional benefit incurred by the hydrophilic PEG part which helps evade opsonization. Further, with an increase in molecular weight of PEG, a slower release pattern can be obtained and the stealth nature of PEG-PLGA can be varied. Though RT PEG-PLGA showed better pharmacokinetic parameters when compared to RT PBS, it is of minor importance as RT needs to enter and remain in the brain for a longer time.

Brain distribution

The C_{max} of RT in the brain achieved after i.v administration of RT PEG-PLGA was more (1058 vs 421 ng/ml) when compared to the concentration RT in the brain achieved after i.v administration of RT PBS. After being encapsulated in the PEG- PLGA polymer, higher concentration (C_{max}= 1058 ng/ml) and longer circulation time of RT ($t_{1/2}$= 17.7 h) was observed than the free form (C_{max}= 421 ng/ml and $t_{1/2}$= 5.70 h) which might be possible because of the stealth effect provided by PEG. Opsonin proteins are present in the bloodstream and bind with conventional nonstealth nanoparticles allowing macrophages of the

reticuloendothelial system to easily recognize and remove the drug before the therapeutic concentration is achieved [21]. To prevent opsonization hydrophilic coatings have been widely used as it prevents the adsorption of opsonin protein via steric repulsion force thereby promoting longer circulation. Since PEG is a hydrophilic surfactant, it inhibits the P-glycoprotein efflux pump and also, enhances uptake by brain endothelial cells by covalently coupling with apolipoprotein E, A-1. Thus, it is evident that the hydrophilic PEG corona present attached to the PLGA has enhanced the permeation of RT across the blood-brain barrier. Secondly, the PEG coat can solubilize the endothelial cell membrane lipids and increase its membrane fluidization thereby enhancing the passage of RT PEG- PLGA. Further, the small size of the nanoparticles and the existence of a high concentration gradient across the blood capillaries could have also enhanced the permeation of RT PEG- PLGA across the blood-brain barrier.

The ratio of RT concentration in brain and blood was calculated at different time points following i.v administration of RT PEG-PLGA and RT PBS. At all time points, the concentration of RT in the brain was high after administration of RT PEG- PLGA. Apart from calculating the brain/ blood ratio, the percentage change (increase) in RT brain- blood ratio (α) was calculated at various times intervals as shown in Fig. 6. The α values indicate an initial increase from 36% (0.25 hr) to ~ 56% (1hr) possibly due to the entry of higher amounts of RT PEG- PLGA. The α value decreased from ~ 56% (at 1 hr) to ~ 41% (at 2 hr). But again, the, α value gradually increased from ~ 41% to ~ 61% (8 hr) indicating the increased $T_{1/2}$ of RT and redistribution of RT from the body to the brain. Hence, it can be confirmed that the PEG coat, apart from increasing the permeability to the brain, would also promote redistribution to the brain and increase its retention in the brain by providing a stealth nature to the nanoparticles. Though it is a well-established fact that tween 80 coated nanoparticles have shown promise in enhancing the permeation of various drugs across the blood, it has not shown industrial applicability citing the presence of tween 80 in the formulations. As the usage of PEG has wide industrial applicability, PEG conjugated polymers like PEG-PLGA, PEG- PCL, etc can hold good promise in enhancing the concentration of drugs across the blood-brain barrier.

Pharmacodynamic studies

It is purported that this positive interaction of nanoparticles with neuronal and astrocyte membranes would not only provide integrity to the membrane but would also help restore the disrupted BBB.

Scopolamine, a muscarinic cholinergic antagonist, is known to cause cognitive impairments. Studies have recommended that the administration of scopolamine specifically impairs the development of spatial navigation strategies, hence affecting acquisition rather than recall or memory consolidation. Scopolamine effects central brain mechanisms underlying spatial learning, impairment of inhibitory avoidance behavior following disturbed amyloid cholinergic functions. Owing to these rationales and to assess the effectiveness of RT PEG-PLGA, screening of learning and memory was done through measuring transfer latency in elevated plus maze and acquisition time in Morris water maze test in scopolamine challenged rats [18]. An elevated plus-maze consisting of two open and two enclosed arms was used for an evaluation of memory. Time taken for rats in the plus-maze escaped from the open arm

to the enclosed arm was recorded. Rats often feel uncomfortable in open and elevated spaces. The Morris water maze is one of the most effectively used screening models in behavioral neuroscience to investigate spatial learning and memory. Morris water maze learning is thought to rely extensively on the hippocampus and involves several major neurotransmitter systems. One such major neurotransmitter system of great importance is the cholinergic system. There was a significant decrease in learning and memory function after cognitive impairment through dysfunctioning of cholinergic transmission by scopolamine which was evidenced by increased transfer latency and acquisition trial in an elevated plus-maze and Morris water maze test. RT at a dose of 2 mg/kg significantly improved both learning and memory was concluded by observing a reduction in transfer latency and acquisition time after cognitive impairment that was induced by 0.5 mg/kg scopolamine. But when compared to RT PBS, RT PEG- PLGA showed better-improved learning and memory enhancement skills. Further, the acetylcholinesterase levels were found to be highest for group 4 which served as scopolamine administered positive group, and least for group 2 which was administered with RT PEG- PLGA. Hence it can be strongly concluded that RT PEG-PLGA are highly efficient in permeating the blood-brain barrier and delivering the drug, RT in a controlled fashion to increase memory learning skills.

Histopathological analysis

The histology of the sections was examined using a trinocular light microscope. The histology of group 4 which received scopolamine showed marked alterations in terms of increased neuronal loss, vacuolated cytoplasm, ghost cells, and hemorrhages (Fig. 7c). The photomicrographs of rats which were treated with RT PBS (Fig. 7a) and RT PEG-PLGA (Fig. 7b) did not show any signs of neuronal degeneration, vacuolization/ spongiosis, and inflammation. This indicates that RT PBS and RT PEG- PLGA not only reduce the scopolamine-induced neuronal degeneration but also don't induce inflammation. The group of rats which served as control (Group 3) did not show any neuronal degeneration or any inflammation (Fig. 7c). Thus, it can be envisaged that RT PEG- PLGA can effectively reverse scopolamine-induced neuronal damage and does not induce any inflammation in the brain.

5. Conclusion

The results indicated PEG- PLGA polymer as a good polymer for the encapsulation and delivery of RT across the blood-brain barrier for Alzheimer's disease. The smaller size of the nanoparticles along with the presence of the hydrophilic PEG group has assisted in enhancing the concentration of RT in the brain which has consequently even improved the memory learning skills of the animals. As PEG- PLGA nanoparticles offer a promising role in enhancing the concentration of drugs across the brain, attempts can be made to enhance the concentration of other anti-Alzheimer's drugs, drugs for treating cerebral malaria, glioma, etc can be attempted.

Declarations

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Declarations

Conflict of Interest The authors declare that there is no conflict of interest

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Figures

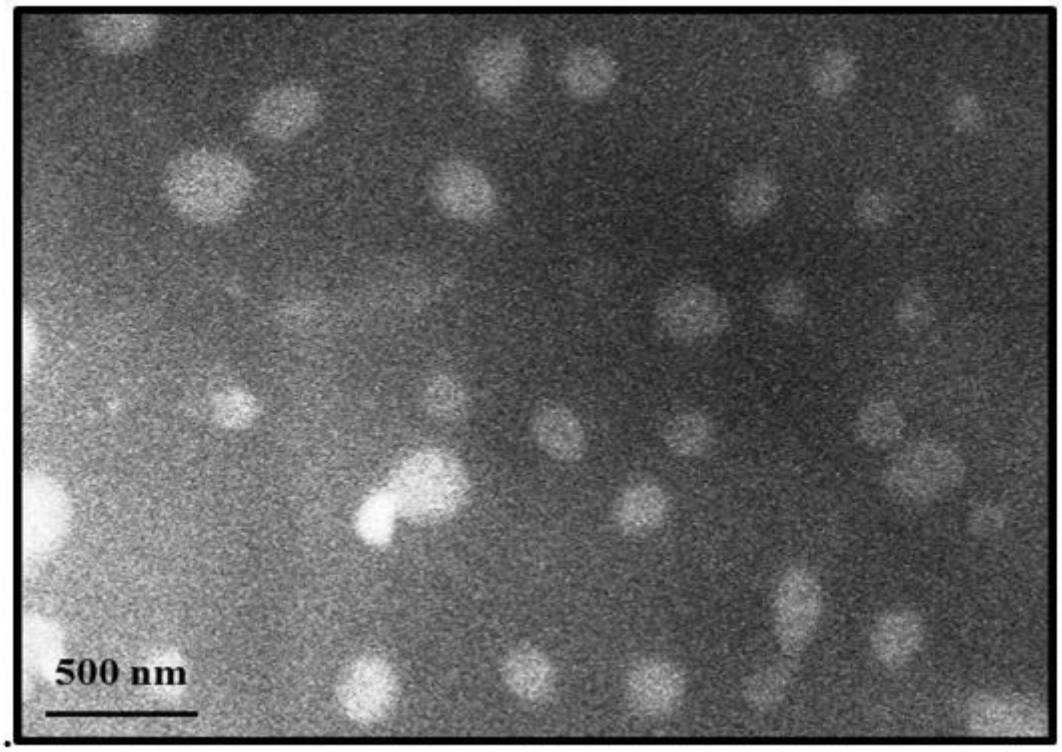

Figure 1

SEM image of RT PEG- PLGA nanoparticles

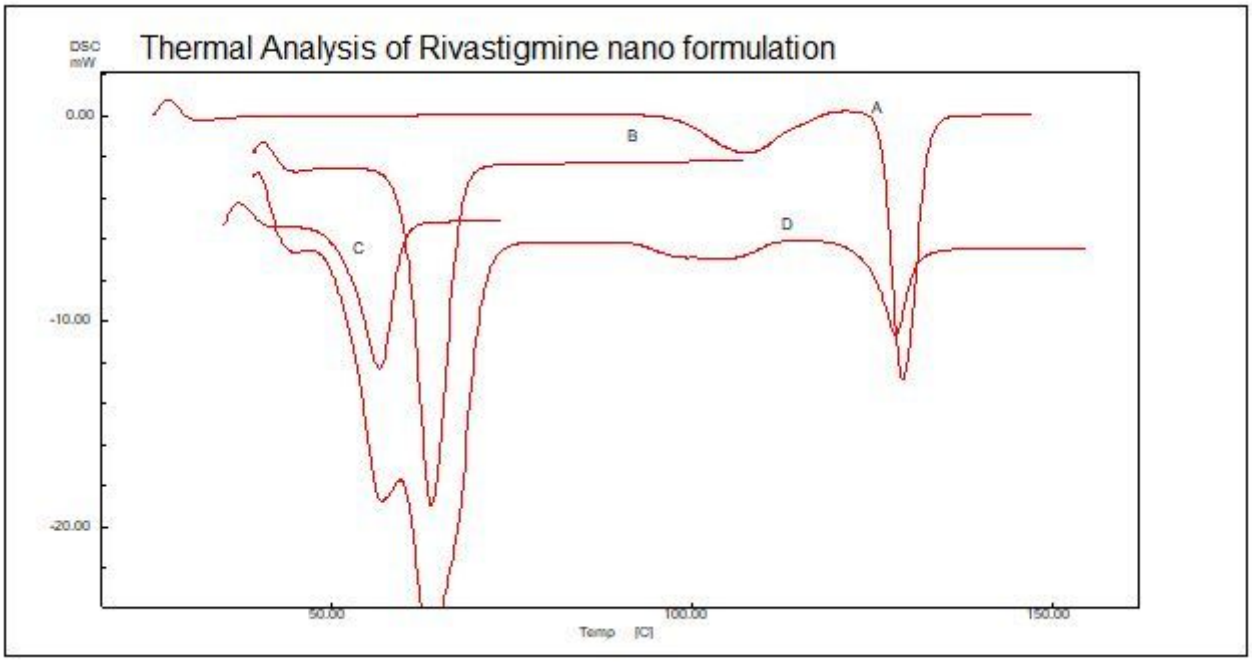

Figure 2

Thermal analysis of A- RT pure, B- Pluronic F 68, C- PEG-PLGA, and D- RT PEG- PLGA nanoparticles

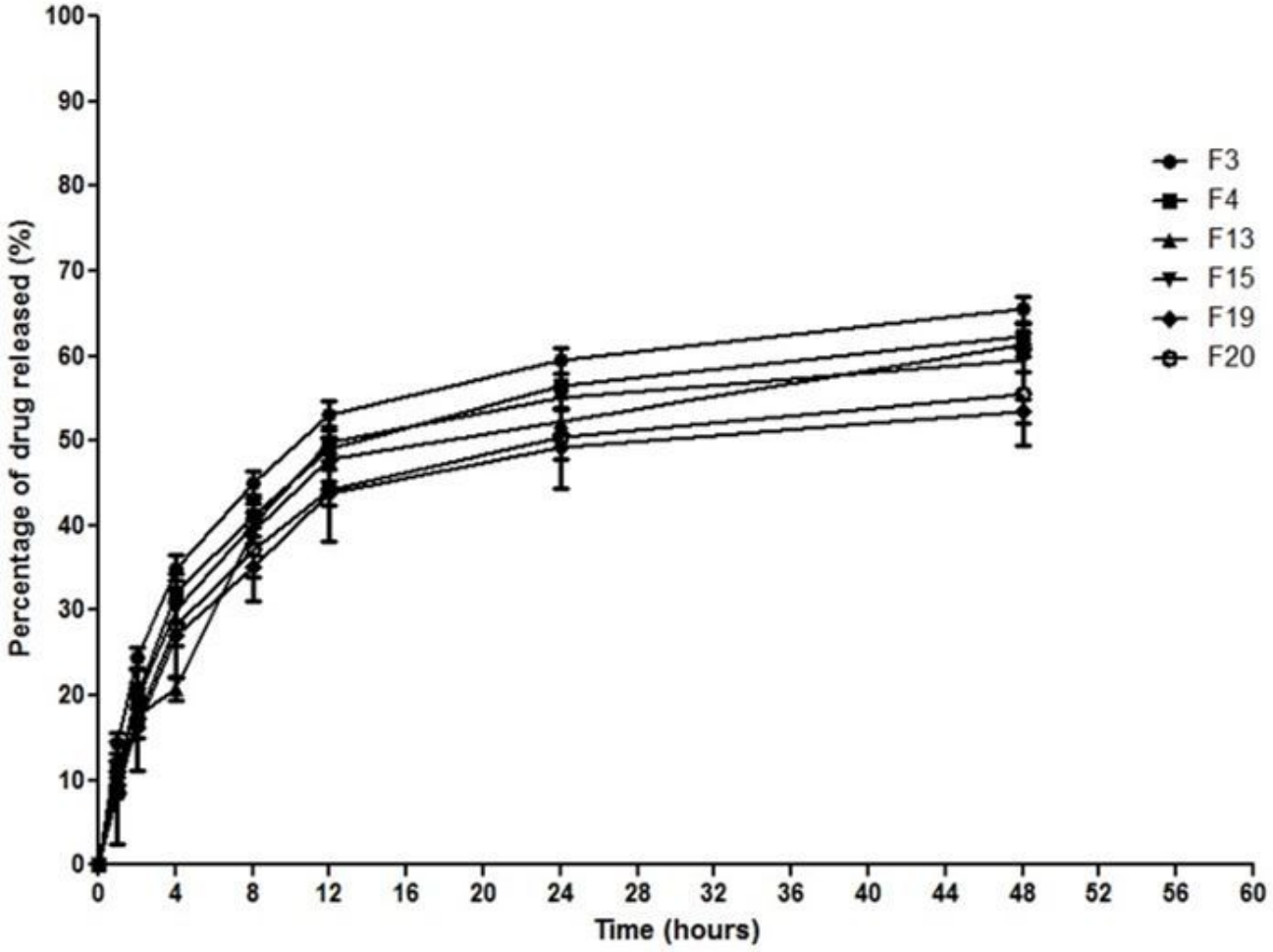

Figure 3

In vitro drug release profile of RT in pH 7.4 PBS

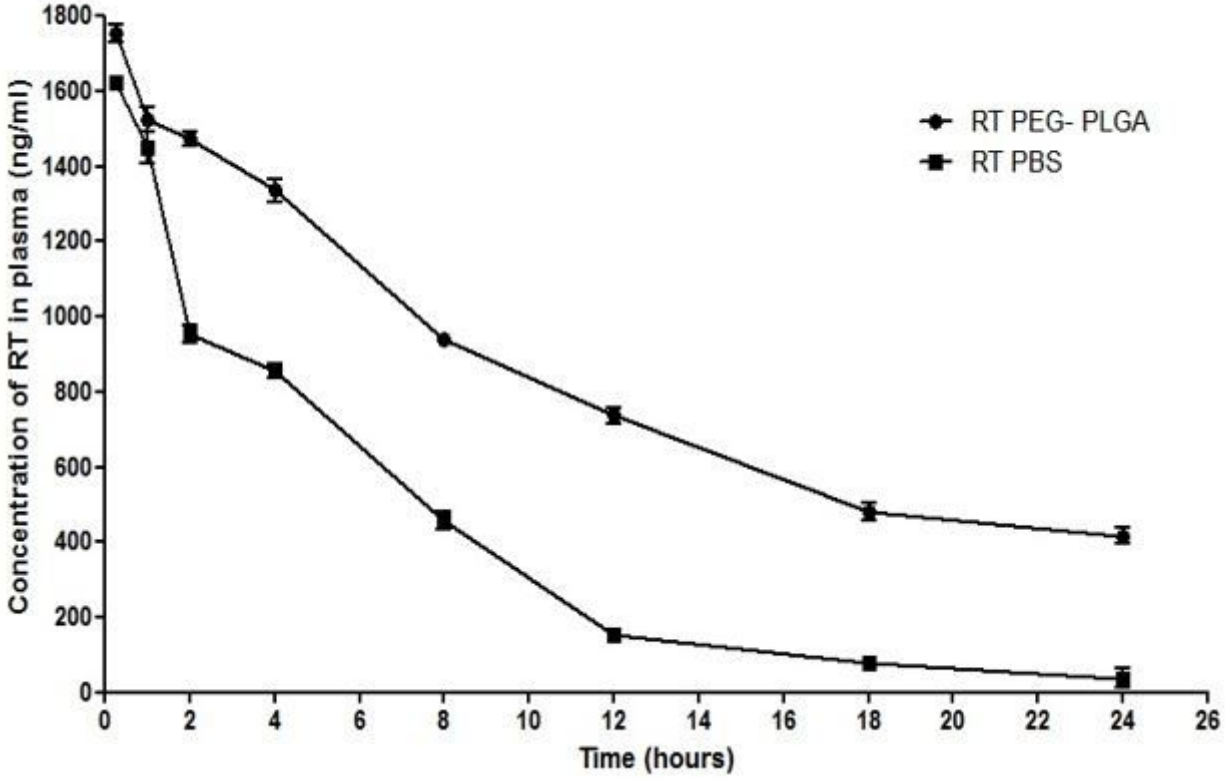

Figure 4

Concentration of RT in plasma at various time intervals

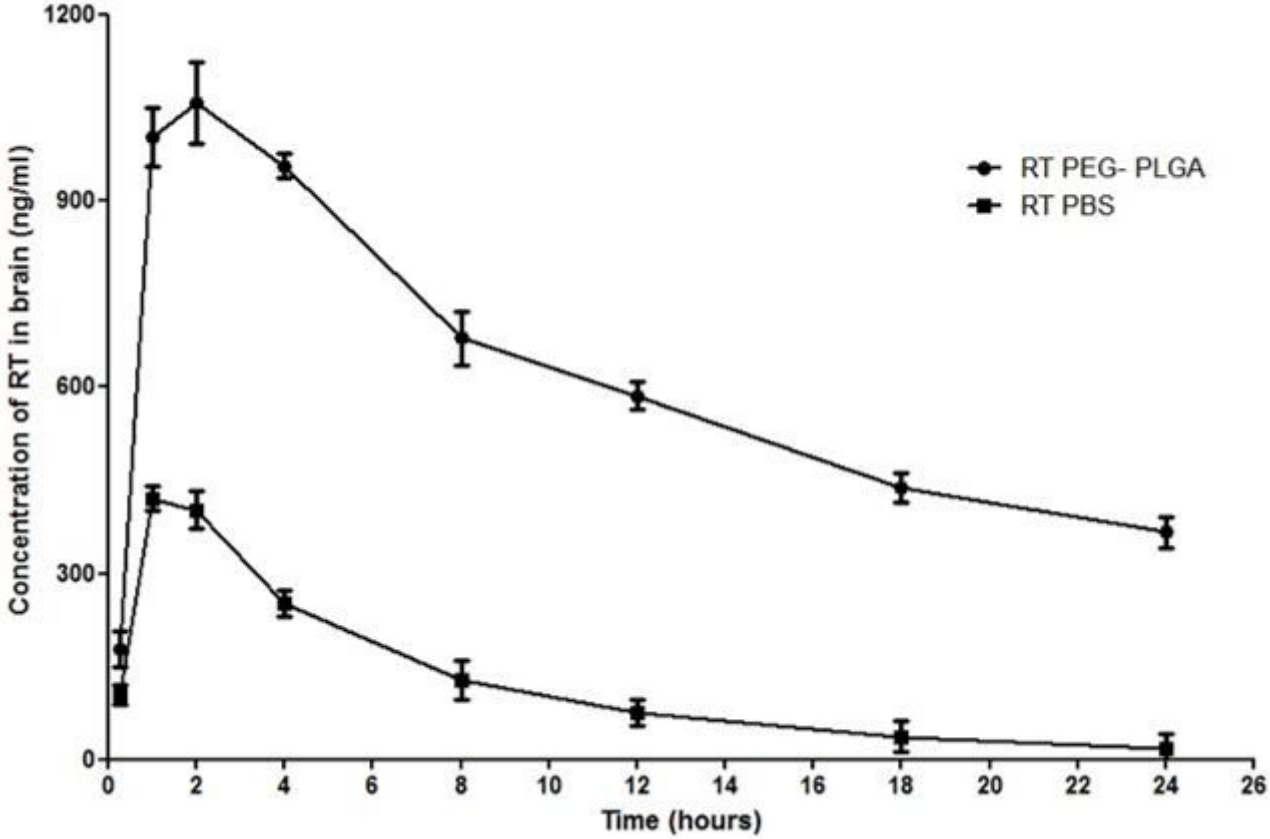

Figure 5

Concentration of RT in the brain at various time intervals

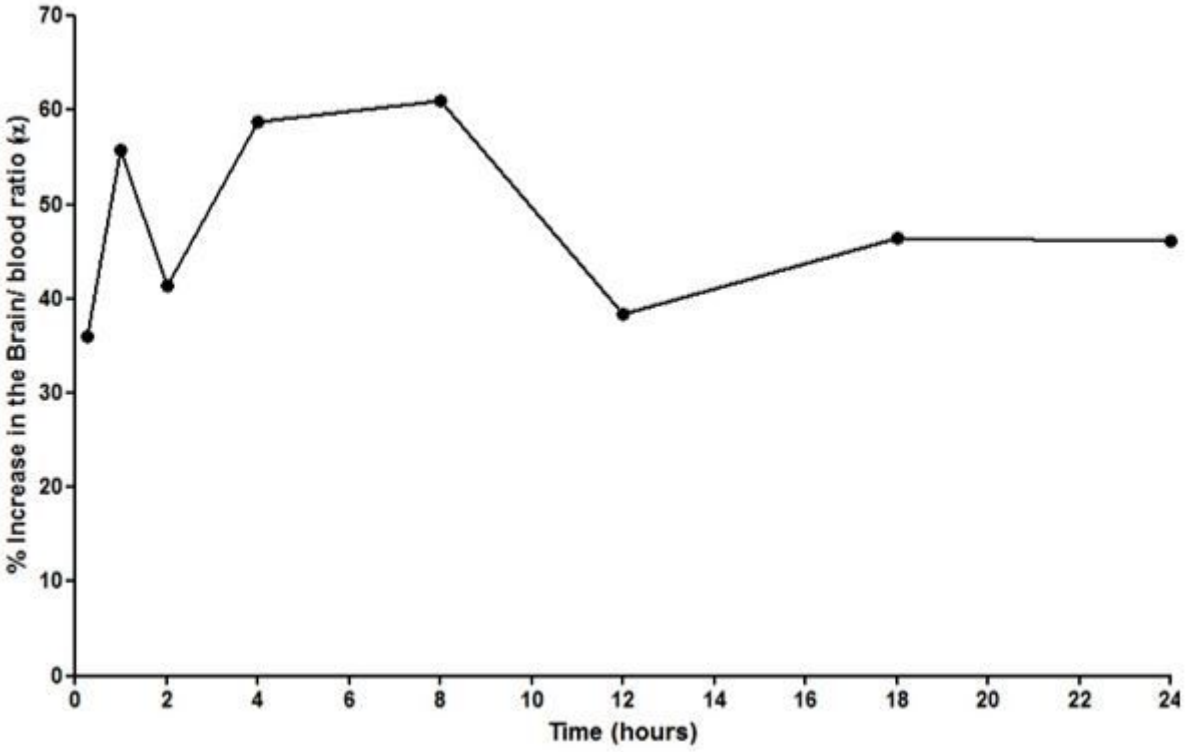

Figure 6

Percentage increase in the brain/ blood ratio at different time intervals

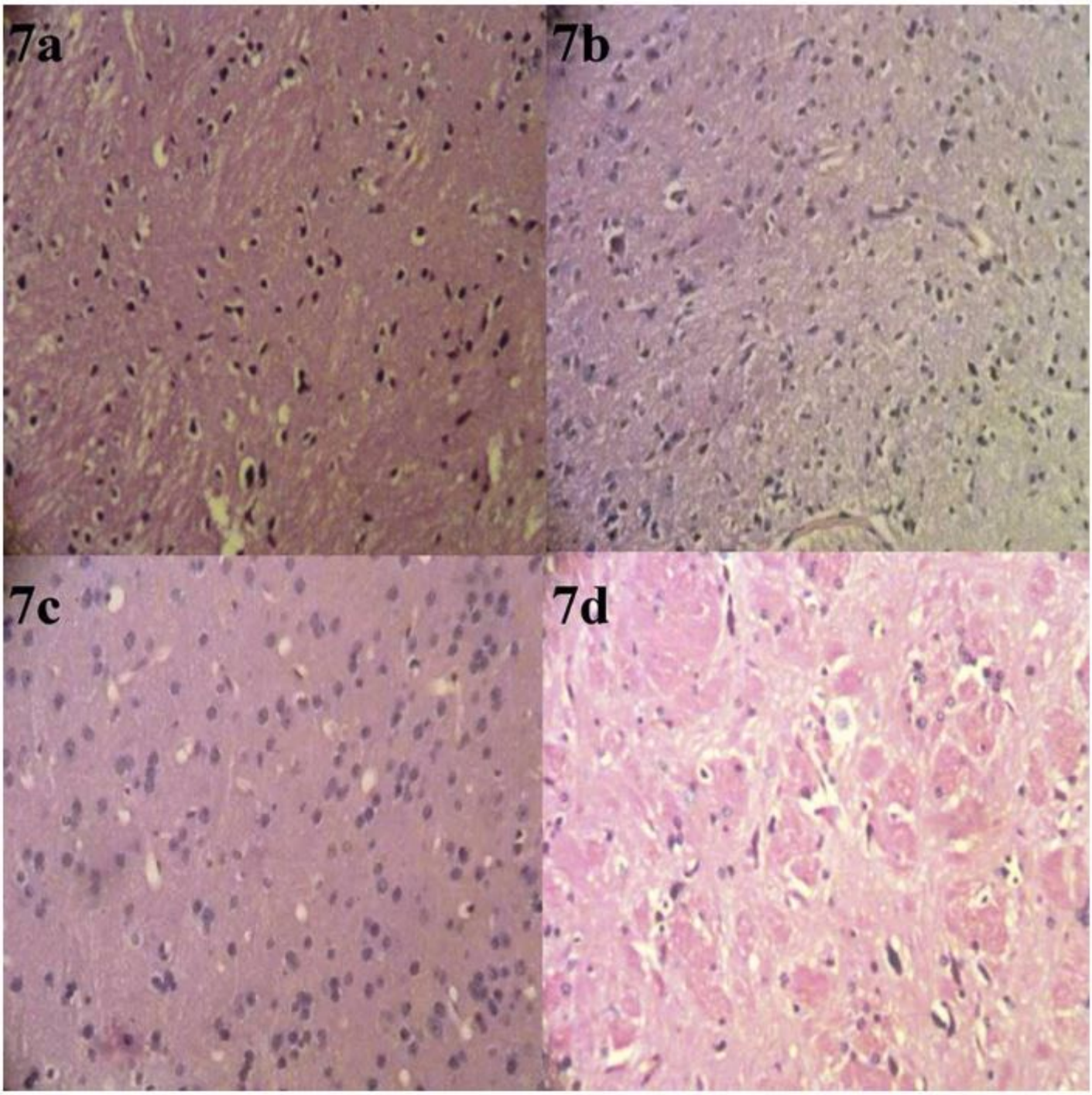

Figure 7

Photomicrographs of brain sections of rats: RT PBS (a), RT PEG-PLGA (b), control (c), and positive control (d)