WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 7, 2158-2172. Research Article ISSN 2277– 7105

EFFECT OF PLGA-CHITOSAN-TWEEN 80 NANOPARTICLES ON BEHAVIOR AND HISTOPATHOLOGY OF HIPPOCAMPUS OF WISTAR RATS

Kinjal C Patel* and Dr. Divakar Goli

Department of Pharmaceutics Acharya & B M Reddy College of Pharmacy, Soldevanahalli, Hesaraghatta Main Road, Bangalore-560107, Karnataka, India.

Article Received on
12 May 2015,

Revised on 07 June 2015,
Accepted on 30 June 2015

***Correspondence for Author**
Kinjal C Patel
Department of Pharmaceutics Acharya & B M Reddy College of Pharmacy, Soldevanahalli, Hesaraghatta Main Road, Bangalore-560107, Karnataka, India.

ABSTRACT

Present study aimed to evaluate effect of PLGA-Chitosan-Tween 80 nanoparticles on the behavior and histology of hippocampus of Wistar rats designed to ascertain whether the nanoparticles improve memory, cognitive deficits and prevent degeneration of hippocampus region of rat brain under AD. This research was carried out using 40 Wistar rats. The animals were divided into four groups. Group I control, II positive control, III standard (drug treated) and IV PLGA-CS-Tween 80 NPs treated group. Animals were evaluated for behavioral study on 0, 7, 14, 28, 36 and 42th day by elevated plus maze paradigm. After 42th day, animals were humanly sacrificed using chloroform and then the brain tissues were fixed immediately in Bouin's fluid and processed further through the routine tissue processor. The stained samples were examined by means of light microscope for histological changes. Behavioral study demonstrated that Retention Transfer Latency (RTL) was decreased in the groups IV and III gradually compare to group II rats. Prepared nanoparticles have been demonstrated improvement in memory and cognitive functions like standard drug in behavioral study. Histology examination showed loosely packed pyramidal cells, neuritic plaques and neurofibrillary tangles, which were indications of neurodegeneration in the group II. However, the group III and IV showed tightly packed pyramidal cells, less number of neuritic plaques and neurofibrillary tangles, which concluded that PLGA-Chitosan-Tween 80 NPs have significant effect on memory, cognitive functions and histopathology compared to group II. The behavioral study and histopathology study supports the performance of NPs *in vivo* into AD model.

KEY WORDS: PLGA-Chitosan-Tween 80 nanoparticles, Alzheimer's disease, behavioral study, Histopathology, Brain targeting.

INTRODUCTION

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders causing dementia among the elderly people. Oxidative stress and amyloid β are considered as the major etiological and pathological factors in the initiation and promotion of neurodegeneration in AD. The neuropathological features of AD include neuronal loss, synaptic depletion, Hirano bodies and granulovacuolar degeneration.[1-3]

AD is 1.5 times more common than stroke or epilepsy and is as common as congestive heart failure.[4] It affects 15 million people worldwide. Moreover, AD has a tremendous negative economic impact amounting to over $ 100 billion a year.[5]

Rivastigmine Tartrate (RT) was approved by the US Food and Drug Administration for the treatment of AD. But its current therapy has many disadvantages, because of its hydrophilicity it could not enter into brain, so necessitating frequent dosing and cholinergic side effects.

However, the targeting of therapeutics to the central nervous system (CNS) is limited by restrictive mechanisms imposed at the Blood Brain Barrier (BBB). Opsonization by plasma proteins in the systemic circulation is also an impediment to cerebral drug delivery.[2,3] Drugs delivery to the brain requires advances in both drug delivery technologies and drug delivery. Drugs that are effective against disease in the CNS and reach the brain via the blood compartment must pass the BBB. The management of brain linked diseases with currently available therapeutic system is very difficult, as inadequate amount of drug reaches the brain, due to extremely lipophilic nature of the BBB. Many strategies have been developed to overcome this problem which includes chemical delivery systems, magnetic drug targeting or drug carrier system such as antibodies, liposomes or nanoparticles.[6-9]

Among these, polymeric nanoparticles have recently attracted great attention as potential drug delivery systems. Due to their small size, NPs penetrate into even small capillaries and are taken up within cells, allowing an efficient drug accumulation at the targeted site over a period of days or even weeks after injection.[10]

Biodegradable NPs can be used successfully for transferring the drug release profile by controlling the polymer degradation. Poly lactide–co-glycolide (PLGA) is one of the well-known biodegradable carrier.[11] Features such as biocompatibility, prediction of biodegradation kinetics, ease of fabrication and regulatory approval has attracted its attention for a variety of biomedical applications.[12,13] Moreover, PLGA matrix can be successfully encapsulated both hydrophilic and lipophilic drugs.[14] PLGA has been used for oral[15, 16] and parenteral[17,18] delivery of drugs. It has been established that Chitosan (CS) is capable of opening the tight junctions of epithelial cells and it can improve the uptake of hydrophilic peptide.[19]

Moreover, Tween 80 coating of chitosan nanoparticles was demonstrated to maximize the translocation of these nanosystems from blood to brain.[20] Therefore, we tried to prepare PLGA NPs with double coating with CS and Tween 80, to acquire advantages of both.

Aluminum is present in small amounts in mammalian tissues, yet it has no recognized physical role. On the contrary, its neurotoxin effect on living organisms is becoming clear, aluminium being implicated as interfering with a variety of cellular metabolic processes in the nervous system and in other systems. Although molecular mechanisms by which aluminium exerts its neurotoxicity remain yet to be established, several pieces of evidence suggest that aluminium can interfere with cellular metabolism in terms of biological stimulation, inhibition, or metal accumulation and compartmentation.[21] Experimentally, it has been demonstrated that chronic exposure to aluminium not only causes neurological signs that mimic progressive neurodegeneration but also results in neurofilamentous changes in the hippocampus, cerebral cortex, brain stem and spinal cord and biochemical changes which are seen in AD.[22] Hence in this research, it was decided to induce AD through oral administration of 100 mg/kg/day of aluminium chloride.

Moving from this background, in the present work we aimed to investigate the potential of RT loaded PLGA-CS-Tween 80 nanoparticles to be administered by IP route and able to overcome the BBB and deliver RT to the brain and analyze NPs activity by behavioral study and histopathology of hippocampus.

MATERIALS AND METHODS

Materials

Rivastigmine Tartrate (RT) was received as a gift sample from SPARC (Vadodara, India). Poly (D,L-Lactide-co Glycolide) (PLGA) [50:50] was purchased from Durate Corporation (Birmingham AL, USA) and was used without further purification. Polyvinyl alcohol (PVA), Tween 80 and Chitosan was purchased from S D Fine Chemicals (Mumbai, India). Aluminium chloride was purchased from Karnataka Fine Chemicals (Bangalore, India). All other chemicals and reagents used in this study were of analytical grade and were used as received.

Methodology

PLGA-CS-Tween 80 NPs preparation method

PLGA-CS-Tween 80 NPs have been prepared by nanoprecipitation method, which was an emulsification-solvent evaporation method. The polymer PLGA (85mg) has been added to 3ml of acetone and was allowed to dissolve with magnetic stirrer (By Remi Equipment Pvt Ltd, Bangalore, India). In above organic solution, RT (4.3mg) was added and allowed to dissolve. This solution has been added with 23 G needle to an aqueous phase of PVA (1%w/v) to form o/w emulsion. Once all the drug and polymer mixture has been added to PVA solution, the contents were allowed to mix for 5 mins with homogenizer (T25 digital Ultra turax by IKA, Germany) at 18000 RPM. The resulting suspension was sonicated for 10 mins with 10s on off with an ultra sonic probe (By Dakshin, Bombay). Immediately after sonication the emulsion was poured into excess of aqueous phase of PVA (1%w/v), CS (0.25 %w/v) and Tween 80 (0.5 %v/v) for solvent evaporation under rapid stirring and coating of CS on PLGA NPs with a magnetic stirrer for 24 h. Then, the nanoparticles were collected by centrifugation and washed 3 times with distilled water. Finally, they were resuspended into 2 ml of cryoprotectant solution (Sucrose (2% w/w)), dried on lyophilizer (Eqsquire Biotech, Germany) and stored at 4°C.[23]

Experimental animals

The subjects used in this research work were 40 adult Wistar rats (male and female both). Wister rats, 200-220g, procured from Bioneeds, Bangalore, were used for investigation. The Institutional Animal Ethical Committee approved the protocol. They were kept in the animal house of Department of Pharmacology, Acharya & B M Reddy College of Pharmacy (Bangalore, India) for seven weeks (normal) at standard environmental conditions

(relative humidity of 60%, 12h-12h light-dark cycle) with sufficient food, water and under a good ventilation in order for the animals (Wistar rats) to acclimatized.

Experimental design

Drug and treatment schedule

Aluminium chloride solution and the PLGA-CS-Tween 80 Nps were freshly prepared at the beginning of each experiment. For oral administration, Aluminium chloride was dissolved in distilled water and for intraperitoneal (IP) administration, prepared nanoparticles were dispersed in normal saline solution (0.9 %w/v). Dose calculated equivalent to 1.5 mg/kg of RT for standard as well as for nanoparticles formulations. Animals were divided into four groups:

Group I: Normal control

Group II: Positive control (Aluminum chloride 100 mg/kg/day p.o)

Group III: Standard (RT 1.5 mg/kg in saline IP + Aluminum chloride 100 mg/kg/day p.o)

Group IV: PLGA-CS-Tween 80 NPs treated (NPs in saline IP + Aluminum chloride 100 mg/kg/day p.o)

Elevated plus maze paradigm study

The elevated plus maze considered of two opposite black open arms (50 cm × 10 cm), crossed with two closed walls of the same dimensions with 40 cm high walls.[24] Acquisition of memory by the rats was tested on the day 0, 7, 14, 21, 28, 35 and 42th. Time taken by the rat to move from the open arm to the closed arm was recorded as Retention Transfer Latency (RTL). Rats were allowed to explore the maze for 20s after recording the reading and were made to return to the home cages. If the rat did not enter the enclosed arm within 90s, it was pushed back into one of the enclosed arm and the reading was recorded as 90s, placing the rat in an open arm assessed its retention of memory. The RTL was noted on day 0, 7, 14, 21, 28, 35 and 42th.[25]

Experimental procedure for histopathology

After oral administration of Aluminium chloride to each group with different formulations except normal control group (I); the animals were sacrificed after the 42th day elevated plus maze study, with the use of chloroform in a closed tight box. Section of the brain was dissected and then fixed in Bouin's solution immediately in order to prevent enzymatic and other postmortem changes that could degrade tissue and also to harden the brain so that it can be sectioned (cut into thin sliced) without tearing. The tissue was processed and stained with

Haematoxylin and eosin (H&E). The stained sections of the hippocampus were examined under the light microscope.[26]

RESULT AND DISCUSSION

Elevated plus maze paradigm study

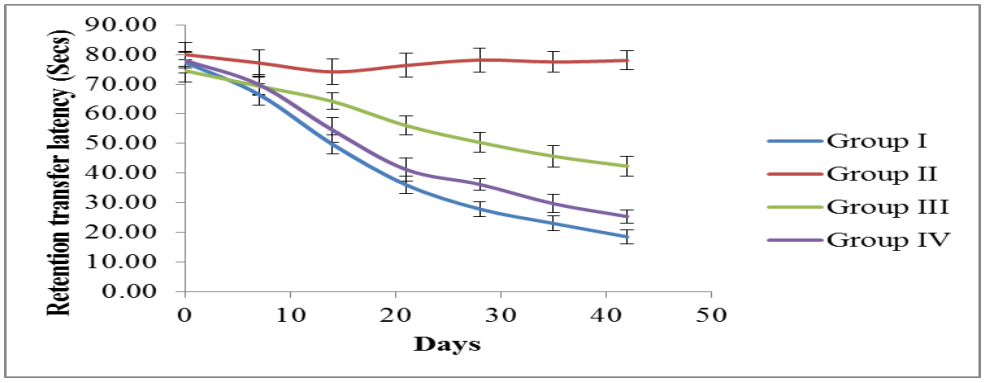


Fig. 1: Comparison of memory retention in various groups of rats using Elevated plus maze paradigm. The values are depicted as mean ± SD (*n* = 6)

In the Elevated plus maze task, we evaluated time taken (Retention Transfer Latency - RTL) by rats to reach from open arm to close arm of maze. The rats from normal, standard and formulation treated group entered the closed arm quickly and RTL found to be decreased. In contrast, Aluminium chloride treated rats performed initially well followed by poorly trough out the experiments. It demonstrates that the chronic administration of aluminum chloride induced marked memory impairment. Regular administration of standard and formulations with Aluminum chloride decreases the RTL compared to positive control group. Groups arranged according to RTL: Group I< Group IV< Group III< Group II.

Histopathology of Hippocampus

According to Brodal, the functions of certain learning and memory have been associated with different areas of the brain like the hippocampus and cerebellum. While the hippocampus is associated with memory of new words, faces, place and event, cerebellum has been associated with memory of learning new skills like playing an instrument etc.[27]

The hippocampus as a whole has the shape of a curved tube, which has been analyzed variously to a seahorse, a ram's horn (*Cornu ammonis*, hence the subdivision CA1 through CA3 and CA4), or a banana. It can be distinguished as a zone where the cortex narrows into a single layer of densely packed pyramidal neurons 3-6 cells deep in rats, which curl into tight U shape; one edge of the "U", field CA4, is embedded into a backward facing strongly flexed

V- shaped cortex, the dentate gyrus.[28] The hippocampus is a major component of the brains of humans and other mammals. It belongs to the limbic system and plays important roles in the consolidation of information from short-term memory to long-term memory and spatial navigation. Like the cerebral cortex, with which it is closely associated, it is a paired structure, with mirror-image halves in the left and right sides of the brain. In humans and other primates, the hippocampus is located inside the medial temporal lobe beneath the cortical surface. It contains two main interlocking parts: Ammon's horn and dentate gyrus. There is now almost universal agreement that the hippocampus plays some sort of important role in memory; however, the precise nature of this role remains widely debated.[29- 31]

Manuela *et al.*, studied on the quantification of the neuronal density in the four specific areas of the hippocampus (CA1-CA4) of AD brains, as compared to an age matched control group, by using Nissl staining technique. In that study they demonstrated and confirmed a significant decrease in neuronal density of hippocampus in AD, as compared to an age-matched control group. Moreover, they come to on conclusion that the decrease of hippocampal neuronal density was more prominent especially at the CA1 and CA3 hippocampal areas.[32] As the reference of this study in the present study we compared only CA1 and CA3 regions of hippocampus for comparative study between different groups.

Group I: Normal control

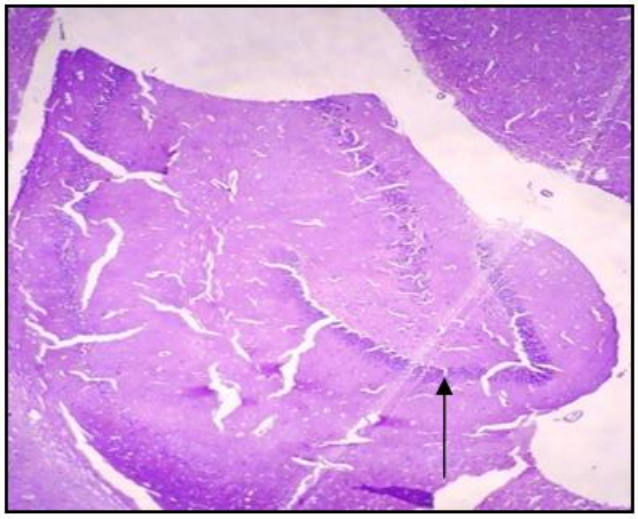


Fig. 2: Section studied from hippocampus shows densely packed pyramidal cells in both CA1 and CA3 layers (H&E; mag. x 50).

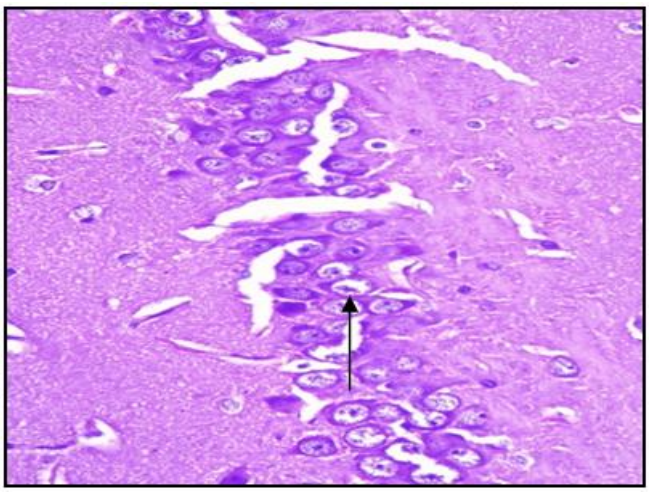


Fig. 3: The CA3 region shows intact pyramidal cell (arrow) in tight clusters (H&E; mag. x 400)

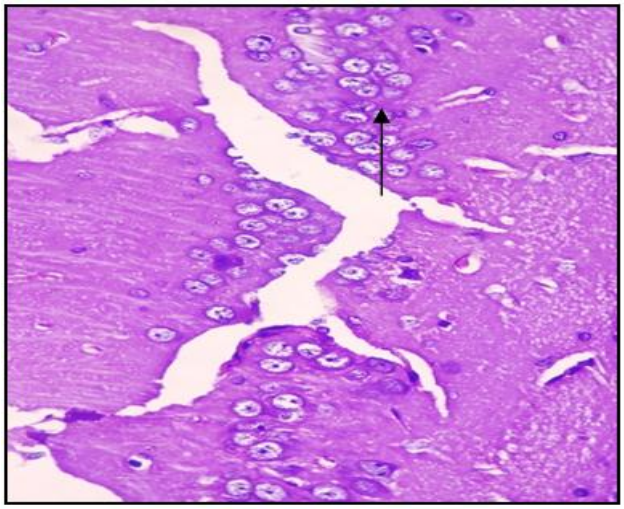


Fig. 4: The CA1 region shows intact pyramidal cells (Arrow) along with intact neurophil fiber. (H&E; mag. x 400)

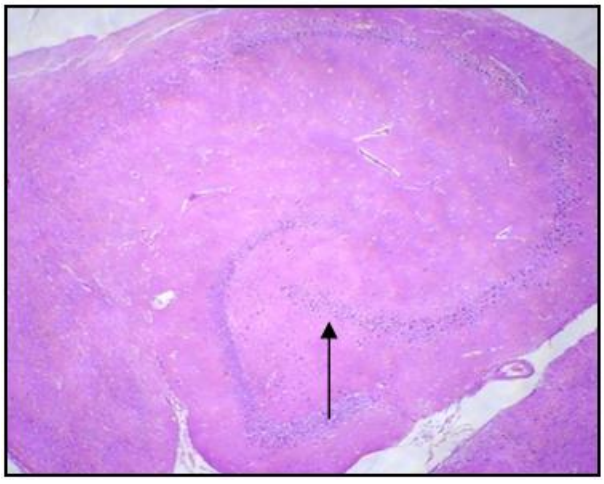
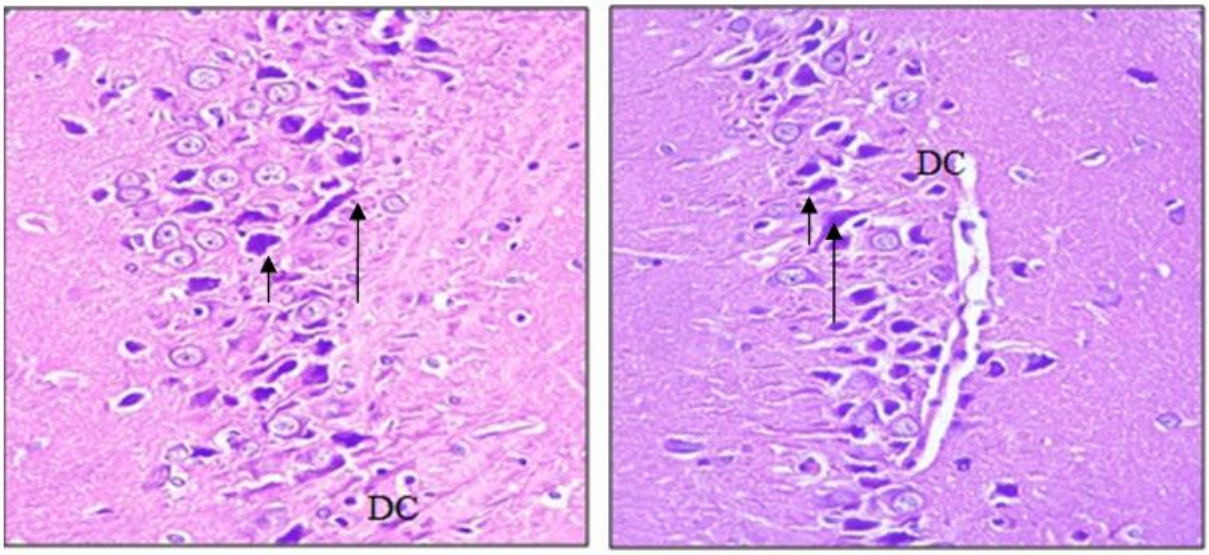


Fig. 5: Section studied from the hippocampus shows (arrow) loosely packed pyramidal cells in both CA1 and CA3 layers. (H&E; mag. x 50)



(A) The CA3 region (H&E; mag. x 400) (B) The CA1 region (H&E; mag. x400)

Fig. 6: The CA3 region (Fig. 6A, Arrow) and CA1 region (Fig. 6B, Arrow) shows loss of both pyramidal cells and neurophil fibers along with neuritic plaques (Short arrow) and neurofibrillary tangles (Long arrow). Some of the pyramidal cells show degenerative changes. (DC – Distorted cells)

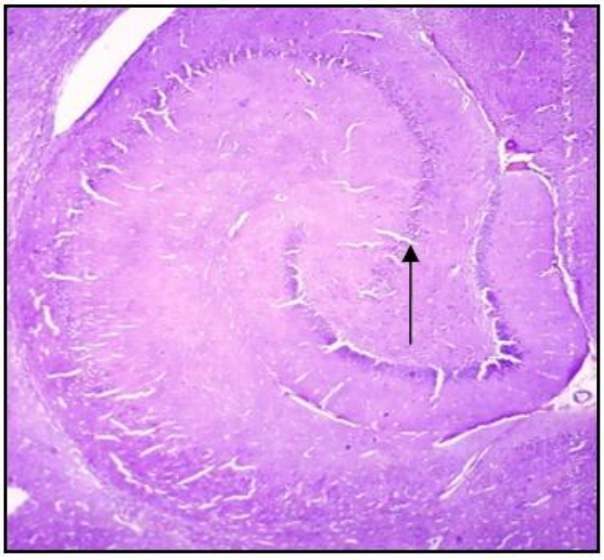


Fig. 7: Section studied from the hippocampus shows densely packed pyramidal cells in both CA1 and CA3 layers (Arrow) (H&E; mag. x50)

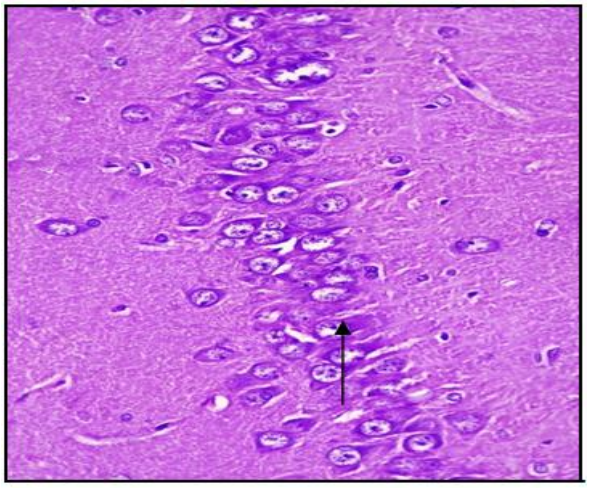


Fig. 8: The CA3 region shows intact pyramidal cells in tight clusters (Arrow). The interconnected neurophil fibers in CA3 region appear intact. (H&E; mag. x 400)

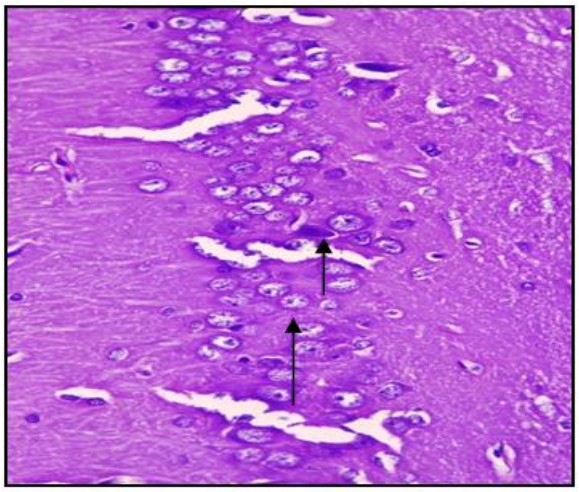


Fig. 9: The CA1 region shows intact pyramidal cells (Long Arrow) along with few neuritic plaques (Short Arrow). (H&E; mag. x 400)

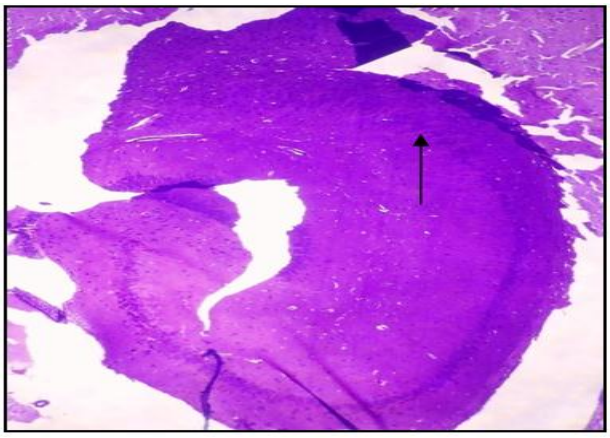


Fig. 10: Section studied from the hippocampus shows loosely packed pyramidal cells in both CA1 and densely packed pyramidal cells in CA3 layers (Arrow). (H&E; mag. x 50)

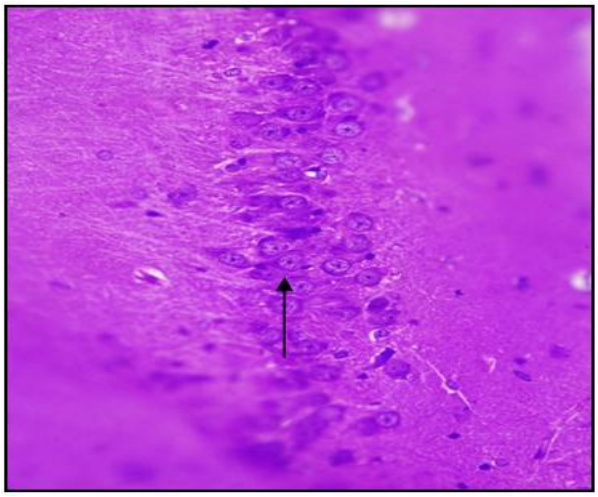


Fig. 11: The CA3 region shows intact pyramidal cells in tight clusters (Arrow). The interconnected neurophil fibers in CA3 region appear intact.
(H&E; mag. x 400)

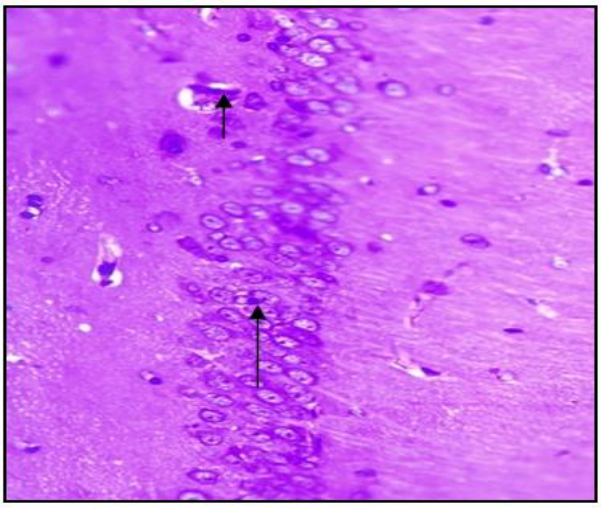


Fig. 12: The CA1 region shows intact pyramidal cells (Long Arrow) along with few neuritic plaques and few neurofibrillary tangles (Short Arrow). (H&E; mag. x 400)

Group II (Fig. 5, 6A and 6B) showed marked cell distortion with high level of degeneration in the cell. In the Figs. 5 and 6 the pyramidal cells packed loosely as compare to Group I (Figs. 2, 3 and 4), Group III (Figs. 7, 8 and 9) and Group IV (Figs. 10, 11 and 12).

Muller *et al.*, who suggested that aluminium might have a role in the pathogenesis of AD although based on circumstances.[33] According to Crapper *et al.*, aluminium concentration was elevated in neurons containing neurofibrillary tangles and perhaps within senile plaques, however, aluminium might accumulate in neurons secondarily to intracellular degenerating changes and the neuropathological and behavioral changes following the aluminium exposure

were similar to those observed in AD and the neurofibrillary changes observed in AD were found mostly within the cortical and hippocampal neurons.[34] Moreover, Group II (Figs. 6A and 6B) showed neuritic plaques and neurofibrillary tangles in the CA1 and CA3 areas of hippocampus, which are also present relatively in to Group IV (Figs. 11 and 12) and Group III (Figs. 8 and 9), which suggested the possible effect of acute oral administration of Aluminium chloride on the brain of the animal (Wistar rats). Based on this hypothesis the AD model was developed into group II. While groups III and IV have shown significantly less amount of neuritic plaques and neurofibrillary tangles, which were due to standard drug treatment in group III and PLGA-CS-Tween 80 nanoparticles of RT treatment into group IV. Figs. 8 and 11 of groups III and IV showed the CA3 region with intact pyramidal cells in tight clusters. Figs. 9 and 12 showed the interconnected neurophil fibers in CA3 region appear intact. The CA1 region shows intact pyramidal cells along with few neuritic plaques and few neurofibrillary tangles. Which indicated that standard drug and nanoparticles of RT showed anticholinergic activity of RT and suppress the progression of AD into aluminium chloride treated animal model (Wistar rats).

CONCLUSION

RT loaded PLGA-CS-Tween 80 NPs have been prepared by nanoprecipitation method, which was an emulsification-solvent evaporation method. Effect of PLGA-CS-Tween 80 NPs on behavioral study and histopathology of hippocampus of Wistar rat was observed. In behavioral study by Elevated plus maze paradigm, demonstrates that the chronic administration of aluminum chloride induced marked memory impairment in group II. Regular administration of standard drug (group III) and drug loaded PLGA-CS-Tween 80 Nps (group IV) formulation with Aluminum chloride decreases the RTL compared to positive control (group II). Furthermore, results of histopathology study of group II showed marked cell distortion with high level of degeneration in the cell and loosely packed pyramidal cells. Histopathology of groups III and IV showed the CA3 region with intact pyramidal cells in tight clusters and intact interconnected neurophil fibers in CA3 region. The CA1 region of group III and IV showed intact pyramidal cells along with few neuritic plaques and few neurofibrillary tangles. Which indicated that standard drug and nanoparticles of RT showed anticholinergic activity of drug RT and suppressed the progression of AD into aluminium chloride treated animal model (rats). Therefore, we concluded that RT loaded PLGA-CS-Tween 80 NPs reduced progression of AD by anticholinergic activity and improve memory, cognitive deficits and prevent degeneration of hippocampus region of rat brain under AD.

ACKNOWLEDGEMENT

Authors are wishing to acknowledge SPARC (Vadodara, India), for providing Rivastigmine Tartrate as gift sample. Authors also thank to Acharya & B M Reddy College of Pharmacy, Bangalore for providing excellent facility to carry out this work.

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