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Cognition enhancement of Punicalagin following intracerebroventricular injection of Aβ $_{(25-35)}$ model of mice

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Abstract

The hallmark in the pathology of Alzheimer's disease is the deposition of amyloid plaques throughout the brain which lead to the deterioration of neuronal function and eventually they die. This damage spreads to hippocampus, region responsible for forming memories. This will ultimately lead to impairment in cognition. Punicalagin a polyphenolic compound found in pomegranate (*Punica granatum*) having proven antioxidant and anti-neuroinflammatory activities in many studies. In recent studies, it was evident that there is a link between Alzheimer's disease and neuroinflammation. In the present study, intracerebroventricular injection of Aβ $_{(25-35)}$ was given to all the three groups of mice except the control group which received only bi- distilled water. Donepezil and Punicalagin at the dose of 10 and 600mg/kg were given to the standard and the test group respectively following icv injection of Aβ $_{(25-35)}$. The results revealed that Punicalagin (600mg/kg) showed significant improvement in cognition as compared to that of the control group.

Keywords: Cognition enhancement, Amyloid beta, Intracerebroventricular injection, Punicalagin, Donepezil

1. Introduction

Alzheimer's disease is one of the most common forms of neurodegenerative diseases and accounts for more than 80% of the dementia cases. Other types of dementias include vascular dementia with Lewy bodies and a group of diseases that contribute to frontotemporal dementia. It leads to the progressive loss of mental, behavioral and cognitive function.

The pathophysiology of AD is related to the injury and death of neurons, initiating in the hippocampus brain region that is involved with memory and learning, then atrophy affects the entire brain. Amyloid beta, (Aβ) is a short peptide that is an abnormal proteolytic byproduct of the transmembrane protein amyloid precursor protein (APP), whose function is unclear but thought to be involved in neuronal development. Amyloid beta monomers are soluble and contain short regions of beta sheet at sufficiently high concentration; they undergo a dramatic conformational change to form a beta sheet-rich tertiary structure that aggregates to form amyloid fibrils. These fibrils deposit outside neurons in dense formations known as senile plaques or neuritic plaques, in less dense aggregates as diffuse plaques, and sometimes in the walls of small blood vessels in the brain in a process called amyloid angiopathy.

Aβ $_{(25-35)}$ is the most effective model and cause impairment in spatial learning of mice[1]. The effect of icv injection can be best observed in case of mice. According to amyloid cascade hypothesis, the APP is normally cleaved by alpha secretase and aberrantly processed by beta and gamma secretase, resulting in an imbalance between production and clearance of Aβ peptide. As a consequence, amyloid beta peptides spontaneously form soluble oligomers and coalesce to form fibril which are insoluble and is in the form of beta-sheet conformation and eventually deposited in diffuse senile plaques.

In recent studies, it has been found that neuroinflammation leads to the Alzheimer's disease pathology. Brain injury leads to the microglial activation which results in the production and release of pro inflammatory cytokines, including interleukins, tumor necrosis factor-alpha and interferon-gamma. All these ultimately lead to the neuronal dysfunction and death.[2]

2. Materials and methods

2.1 Materials

Punicalagin, a byproduct of Pomegranate and Hamilton syringe used for icv injection were purchased from Sigma Aldrich, Bangalore, India. 90% Ethanol was purchased from Nice chemicals, Ernakulum, Kerala, India. Povidone iodine (Betadine) was purchased from Divine Pharmaceuticals, Kottayam, Kerala, India. Oxytetracycline was purchased from Mariya medicals, Kottayam, Kerala.

2.2 Methods

2.2.1 Animals

Male mice were obtained from the animal house of Department of Pharmaceutical Science, RIMSR, Puthupally, Kottayam, Kerala, India and approval for conducting animal experiment were obtained from the Animal Ethical Committee with the approval no: **IAEC Reg. No: MGU/DPS/IAEC/2016/M. Pharm – 11.** Male mice weighing between 20-25g were used. Animals were maintained in the registered animal house of Department of Pharmaceutical Science, RIMSR, Puthupally under controlled environment (23±2ºC, 12 h-light/dark cycle, free access to food and water *ad libitum.*

2.2.2 Acute toxicity studies

The dosage for the present study was selected based on the acute toxicity study conducted by Rajalakshmi and Devaraj (2001)[3], the safe dose of Punicalagin was found to be 600mg/kg.

2.2.3 Amyloid beta

The β- amyloid (25-35) peptide fragment (Aβ) was dissolved in distilled water at a concentration of 2mg/ml and was then incubated for 4 days at 37ºC to allow the formation of fibril like structures[4] before icv administration. Mice were anesthetized with 60 mg/kg of Thiopental and placed in a stereotaxic frame.

2.2.4 Surgical Procedure

All the animals were kept on overnight fasting on the day prior to surgery. All the equipment used during the surgical procedure was sterilized by washing with 90% ethanol before every use. Mice were administered with oxytetracycline injection IM one hour prior to the surgery to prevent infections.

Mice were anesthetized with 60 mg/kg of Thiopental and placed in a stereotaxic frame.[1] Mice were kept in a supine position on a surgical board and a cotton ball was placed under the lower jaw to prevent any obstruction to airflow during the surgery. The fur was removed from the head using scissors and the surgical area was wiped with cotton dipped in alcohol and later with betadine solution. A midline sagittal incision was made in the scalp using a scalpel blade. The excess blood was removed with cotton and cleaned with normal saline. The coordinates for drilling holes were identified using a stainless steel scale. Holes were drilled in the skull over the lateral ventricle using the following coordinates 1mm lateral to bregma, 1mm posterior and 2.5mm deep from the pial surface All injections were made using 10µl Hamilton syringe. The needle of the micro syringe was placed 3.8mm beneath the surface of the brain.

2.2.5 Post Surgical Care[3,5]

Oxytetracycline was given once daily IM, for the next 3 days and Pentazocine was given for the next 2 days once daily subcutaneously. The hind and forelimbs of the animals were cleaned with alcohol every 12h to prevent any fecal contamination of wound and the bedding was changed every day. Food and water was supplied after 24 hour of surgery. Betadine ointment was applied every 12 hour for the next 2 days to prevent any chances of infection and faster wound healing.

On day 26^{th}, 27^{th} and 28^{th}, Morris water maze, Radial arm maze and Marble burying test were conducted to explore the spatial learning, short term memory and exploratory behavior respectively.

2.2.6 Morris Water Maze:

It was developed by Richard Morris at the University of St Andrews in Scotland and first described in two publications in the early 1980s.[6,7] This method is now widely used to assess spatial learning and memory. Male mice were placed in a circular pool of water which was made opaque by adding milk to the water and were allowed to swim to reach the platform. The platform was placed in a fixed quadrant. This training was continued for about 7 days pre-operatively. Donepezil and Punicalagin at the dose of 10mg/kg and 600mg/kg respectively were administered two days after the surgery. After 16 days of recovery period, the animals were again allowed to swim to find the platform. The time taken to reach the platform was noted.

2.2.7 Radial Arm Maze:

This method was developed by Olton and Samuelson in 1976. This method is employed to assess short term memory. A habituation trial of 5 days was carried out pre-operatively. Firstly, male mice were placed in the center of the radial arm maze. Food pellets were placed at the end of each arm. Donepezil and Punicalagin at the dose of 10mg/kg and 600mg/kg respectively were administered two days after the surgery. After 16 days of

recovery period, the animals were again placed in the center of the radial arm maze and allowed to visit the arms. Revisiting the arm is considered as an error.

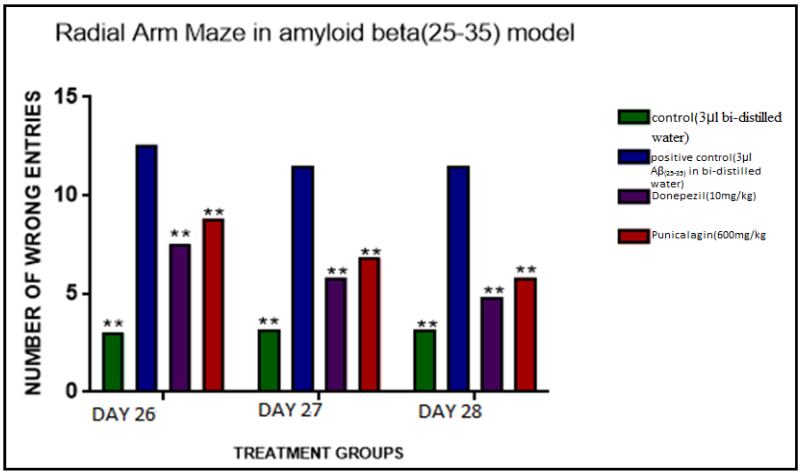
2.2.8 Marble burying task[8]:

Marble burying task is an animal model used in scientific research to depict anxiety and exploratory behavior. Male mice are placed for thirty minutes in a plastic box filled with 5cm depth of wood chip bedding with 10 marbles evenly spaced. After 30 minutes, the number of marbles buried is measured. A marble is considered buried if 2/3 of the marble is covered with bedding.

3. Results

On day 26, the intensity of memory impairment is high in positive control group. Punicalagin and Donepezil showed improvement in cognition as compared to the positive control group. On day 27, the intensity of memory impairment is increased in positive control group. Punicalagin and Donepezil showed improvement in cognition as compared to the positive control group. On day 28, the intensity of memory impairment is aggravated in positive control group. Punicalagin and Donepezil showed improvement in cognition as compared to the positive control group.

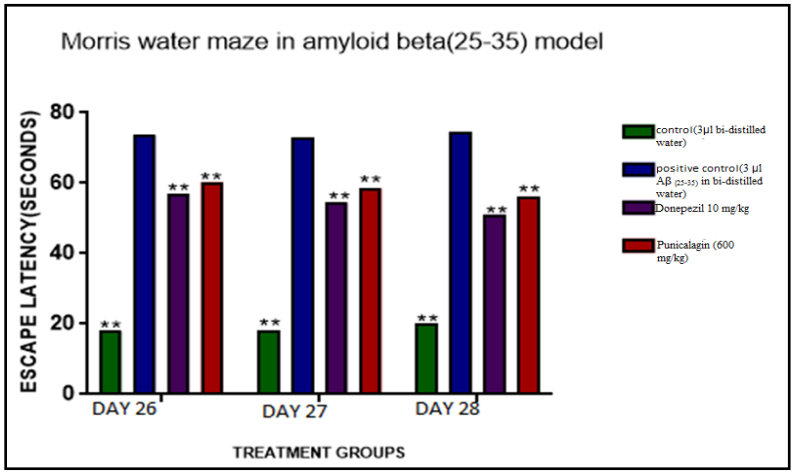
Figure I: The effect of Punicalagin in Radial arm maze of beta amyloid model



Values are presented as mean ± S.E.M by one-way Analysis of Variance (ANOVA) followed by Dunnett's test (N=6), **p<0.01 significant different compared to the positive control.

On day 26, the intensity of impaired spatial learning is high in positive control group. Punicalagin and Donepezil showed improvement in spatial learning as compared to the positive control group. On day 27, the intensity of impaired spatial learning is increased in positive control group. Punicalagin and Donepezil showed improvement in spatial learning as compared to the positive control group. On day 28, the intensity of impaired spatial learning is aggravated in positive control group. Punicalagin and Donepezil show improvement in spatial learning as compared to the positive control group.

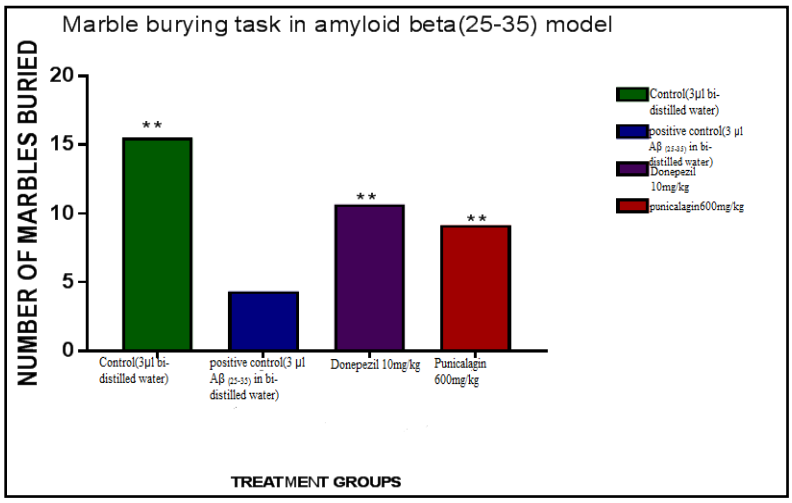
Figure II: The effect of Punicalagin in Morris water maze of beta amyloid model.



Values are presented as mean ± S.E.M by one-way Analysis of Variance (ANOVA) followed by Dunnett's test (N=6), **p<0.01 significant different compared to the positive control.

In figure III, the positive control group shows least exploratory behavior. Punicalagin and Donepezil showed improvement in exploratory behavior as compared to the positive control group.

Figure III: The effect of Punicalagin in Marble burying task of Amyloid beta model.



Values are presented as mean ± S.E.M by one-way Analysis of Variance (ANOVA) followed by Dunnett's test (N=6), **p<0.01 significant different compared to the positive control.

4. Discussion

In amyloid beta $_{(25-35)}$ model, the nootropic activity of Punicalagin is evaluated. It was already proven that single I.C.V injection of Aβ $_{(25-35)}$ has potent amnesic properties in Albino mice especially in short term memories like spatial working memory, spatial learning. In the present study it was found that (Figure: I,II & III), the I.C.V injection of Aβ $_{(25-35)}$ had resulted in an impairment of short term memory compared to Control group which received bi-distilled water instead of Aβ as I.C.V injection.

Aβ $_{(25-35)}$ is the most effective model and cause impairment in spatial learning of mice[1]. The effect of I.C.V injection can be best observed in case of mice.

Current evidence favours the idea that the intracerebroventricular injection of Aβ$_{(25-35)}$ impairs the cognition by showing deficits in place learning, spontaneous alternation and passive avoidance.[4] Amyloid beta is a potential neurotoxic peptide for primary neuronal cortical cells which produces neurofibrillary tangles[9]. Despite the protective role that blood brain barrier plays in shielding the brain, it limits the access to the central nervous system (CNS) which most often results in failure of potential therapeutics designed for neurodegenerative disorders.[10,11] So intracerebroventricular injection allows delivery of drugs directly into the lateral ventricles by bypassing blood brain barrier. The intracerebroventricular injection is safe and can be performed freehand, with mice recovering shortly after injection without any detrimental side effects. This is suitable for rapid injection of a group of animals in a short period of time. [12]

Morris water maze, Radial Arm Maze and Marble burying task were used for the evaluation of spatial learning, short term memory and exploratory behavior respectively.

Morris water maze is designed mainly to study spatial localisation in the mice. The principle behind the study is that the mice could escape from the water by swimming randomly throughout the pool; but in case of normal mice they quickly learn to swim directly towards the platform from any starting position at the circumference of the pool. It is used to assess the spatial learning of the mice. The main parameter used to assess the spatial learning is the escape latency (time taken to reach the platform).

Morris water maze performance has been linked to long-term potentiation (LTP) and NMDA receptor function, making it a key technique in the investigation of hippocampal circuitry.[13] In addition, it has been shown that there is involvement of the entorhinal and perirhinal cortices, as well as involvement of the prefrontal cortex, the cingulate cortex, the neostriatum, and perhaps even the cerebellum in a more limited way.[13] The Morris water maze is primarily a test of spatial learning and reference memory and that remains its principal strength. The concept behind it is that the animal must learn to use distal cues to navigate a direct path to the hidden platform when started from different, random locations around the perimeter of the tank.

Spatial learning refers to the process through which animals encode information about their environment to facilitate navigation through space and recall the location

of motivationally relevant stimuli. Spatial abilities are fundamentally important for navigating the world in order to migrate, avoid dangers such as predators, and to locate biological necessities such as food, shelter, and mates. It was found that certain complex cells called place cells are activated in a selective way when animals are in specific places in a familiar environment.[14]

From our study, it was observed that on day 26, the impairment in spatial learning was found to be high in positive control group, 73±1.11 (**p<0.01). In test group (Punicalagin 600mg/kg), it was found to be 60.3±2.07 (**p<0.01) and in standard group (Donepezil 10 mg/kg), it was found to be 57±7.67 (**p<0.01). On day 27, the spatial learning gets aggravated in positive control groups 73±0.77 (**p<0.01). In test group (Punicalagin 600mg/kg), it was found to be 58.5±2.07 (**p<0.01) and in standard group (Donepezil 10 mg/kg), it was found to be 54.5±2.01. On day 28, the spatial learning gets intensified in positive control groups 74.6±0.88 (**p<0.01). In test group (Punicalagin 600mg/kg), it was found to be 56.16±1.95 (**p<0.01) and in standard group (Donepezil 10mg/kg), it was found to be 51.16±2.38 (**p<0.01). It was evident from this observation that the test compound showed significant cognition enhancement.

Radial arm maze is mainly designed to assess short term memory. The parameter used is the number of wrong entries. Short term allows one to temporarily store and manage information that is required to complete complex cognitive tasks. Task which employ short term memory include learning, reasoning and comprehension.

Marble burying task is used to assess the exploratory behavior of animals. Rodents will also bury non aversive unconditioned objects, such as food pellets and glass marbles[8]. The marble-burying assay was developed to take advantage of this inherent burying behavior to evaluate how many novels, but harmless, glass marbles a rodent would bury. The presence of a novel object (i.e., glass marbles) in a cage may trigger a behavioral response from mice resulting in burying behavior targeted toward that object.

In Morris water maze, Radial arm maze and Marble burying task, when compared to the positive control, Donepezil (10 mg/kg) was found to be more effective than Punicalagin (600mg/kg). Although Donepezil show significant response, Punicalagin shows significant response as that of the control

5. Conclusion

Punicalagin shows significant cognition response as compared to the control.

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