Pelargonidin Improves Passive Avoidance Task Performance in a Rat Amyloid Beta$_{25-35}$ Model of Alzheimer's Disease Via Estrogen Receptor Independent Pathways

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Abstract- Alzheimer's disease (AD) is a disorder with multiple pathophysiological causes, destructive outcomes, and no available definitive cure. Pelargonidin (Pel), an anthocyanin derivative, is an estrogen receptor agonist with little estrogen side effects. This study was designed to assess Pel memory enhancing effects on the a rat Amyloid Beta$_{25-35}$ (Aβ) intrahippocampal microinjections model of AD in the passive avoidance task performance paradigm and further evaluate the potential estrogen receptor role on the memory-evoking compound. Equally divided rats were assigned to 5 groups of sham, Aβ intrahippocampal microinjected, Pel pretreated (10 mg/kg; P.O), α estrogen antagonist intra-cerebrovascular (i.c.v.) microinjected, and β estrogen antagonist (i.c.v) microinjected animals. Intrahippocampal microinjections of Aβ were adopted to provoke AD model. Passive avoidance task test was also used to assess memory performance. Pel pretreatment prior to Aβ microinjections significantly improved step-through latency (P<0.001) in passive avoidance test. In α and β estrogen, antagonists received animals, passive avoidance task performance was not statistically changed (P=0.11 & P=0.41 respectively) compared to Pel pretreated and sham animals. Our results depicted that Pel improves Aβ induced memory dysfunction in passive avoidance test performance through estrogen receptor independently related pathways.

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Keywords: Alzheimer's disease; Hippocampus; Pelargonidin; Memory dysfunction; Passive avoidance test; Estrogen receptor

Introduction

Alzheimer's disease is a brain neurodegenerative ailment and the commonest cause of dementia. It usually affects memory performance, language, problem-solving activities and cognitive learning and disturbs normal personal life. Patients, in the end, stages of the disease would be bed-ridden and require full care services until their early death (1). More than 35 million people around the globe were affected (2). Based on informal data adapted from Iran Alzheimer's Association around 300,000 up to 450,000 people are suffering from the disease. The medial temporal lobe region, such as hippocampus is mostly undergone synaptic changes and neuronal damage during the disease course. It can lead to death within 3 to 9 years post medical diagnosis (2,3). The disease is a leading health care and social problem with few palliative treatments and means of diagnosis but post-mortem brain biopsy (4).

Apart from deranged mechanisms such as mitochondrial dysfunction, mitotic changes, and genetic components, many other pathophysiological defects have also been identified in the course of the disease. The pathophysiology of AD is intricate and includes several neurotransmitter systems and pathophysiological processes. However 3 hallmarks are most prominent which involve β amyloid plaques, neurofibrillary tangles, and neuronal cell death. Several challenging hypotheses have been designed to explain the disease basic cause which are amyloid hypothesis, tau hypothesis, cholinergic hypothesis, oxidative stress hypothesis, glutamatergic hypothesis, cholesterol,

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chronic inflammation and other neurotransmitter deficiencies (5), but misfolded proteins accumulated in the aging brain is the core neuropathological hallmark which results in oxidative and inflammatory damage followed by energy failure and synaptic dysfunction (2,6). Among all molecular defects, the Aβ peptides have long been said to play a pivotal role in the pathogenesis and progression of the disease and " amyloid cascade hypothesis" has received most investigators' attention (7). It appears that cellular adaptive strategy to oxidative stress in response to amyloid beta production is becoming the dominant hypothesis in the literature (8).

Despite noticeable advances in AD's pathophysiology and therapeutic knowledge, only four anticholinesterase drugs plus memantine have been approved by US Food and Drug Administration for symptomatic treatment due to the complicated nature of the disease (9). Accordingly, researchers have turned their focus to new types of therapeutical modalities. Among disparate options, flavonoid-based sources like anthocyanins have received particular focus because of their high oral intake in humans and multiple health promoting effects such as improving motor function, antiviral, vasoprotective, antiangiogenic activity, hippocampal neuroplasticity, proteasome inhibition, protein misfolding and aggregation prevention (10), antiatherogenic properties (11), anti-inflammatory, antioxidant (12), anti-adhesive, estrogenic/antiestrogenic activity, angiotensin-converting enzyme inhibitory properties (13), antihyperglycemic activity (14) and ocular preventive and protective effects (15).

Anthocyanins are water-soluble pigments largely found in red, purple, and blue colored plant tissues (16). Pelargonidin (Pel), an anthocyanin with above-mentioned flavonoid's useful properties plus efficient absorption from the gastrointestinal tract (17), blood-brain accessibility (18), non-genotoxicity, tremendous efficacy, reasonable price (19), neural protection (20), antihyperglycemic activity (21) and anti-thrombosis function (22) grabbed our attention to design an introductory study to investigate the possible memory enhancing role of oral Pel pretreatment in an intrahippocampal Amyloid $Beta_{25-35}$ rat model of AD.

Materials and Methods

Animals

Forty locally bred male Wistar rats weighing 280-320 g were identically divided into five groups and kept at the animal house with free access to standard chow and tap water at 21 ± 2 ºC, relative humidity of 45 ± 15 % and 12 hours light/dark cycle. Passive avoidance task test accomplished from 8 a.m. till 4 p.m. All procedures for care and use of animals conducted in accordance with Tehran University of Medical Sciences regulations and those specified by National Institutes of Health (NIH).

Experimental procedure

Rats were equally divided into 5 groups of sham, $Aβ_{25-35}$ intrahippocampal microinjected (Aβ), Pel pretreated (10 mg/kg; P.O) (Aβ + Pel), α estrogen antagonist intra-cerebrovascular (i.c.v) microinjected (Aβ + Pel + Anti Eα), and β estrogen antagonist intra-cerebrovascular microinjected (Aβ + Pel + Anti Eβ) animals.

Stereotaxic surgery was done under general anesthesia upon intraperitoneal ketamine (100 mg/kg) and xylazine (5 mg/kg) mixture administration. After anesthesia induction, the animal head was symmetrically held in Stoelting stereotaxic instrument to achieve skull flat position. The scalp skin was clean shaved and scrubbed with a solution of 10% povidone-iodine. A midline incision was made. Two burr holes were symmetrically made with a micro drill over the skull at coordinates of -3.5 mm posterior to the bregma, ± 2 mm lateral to the sagittal suture and 2.8 mm ventral to dura matter, based on the rat brain in stereotaxic coordinates (23) for bilateral amyloid $beta_{25-35}$ fragment, vehicle or saline microinjections. Pel (10 mg/kg, Sigma-Aldrich Chemicals, USA) in chromophore as a solvent was orally administered once a day for three days through a stainless steel ball-tipped feeding needle. The last dose was taken one hour prior to the stereotaxic procedure based on the pilot and our earlier studies (21). In Aβ microinjected animals, 2 µl of an $Aβ_{25-35}$ (5 µg/µl) solution prepared in normal saline with pH = 8, preincubated at 37 ºC for 72 hours, bilaterally microinjected into dorsal hippocampus. In sham-operated rats, normal saline was microinjected accordingly. 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP), α estrogen receptor antagonist (Anti Eα) and 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenolas (PHTPP), as β estrogen receptor antagonist (Anti Eβ) were dissolved in dimethyl sulfoxide (DMSO) and diluted with aCSF (Merck Chemical, Germany). Prepared solutions (10 µg/rat) were i.c.v injected at a volume of 5 µl at coordinates -1 mm posterior to the bregma, 1.5 mm lateral to the midline and 4 mm ventral

to dura matter under general anesthesia 30 min post-Pel gavage. $A\beta_{25-35}$ (5 µg/µl) was also bilaterally microinjected into the posterior hippocampus 30 min after i.c.v microinjection of the blockers at the mentioned coordinates. Passive avoidance test was then carried out after surgical recovery by an observer blind to the experimental groups.

Passive avoidance test

Passive avoidance is a fear-motivated test to study long and short term memories in an associative manner. It needs the animal to behave opposite to its innate dark preference. The test was started on day 24 post-surgery using the shuttle-box apparatus consisting of two compartments isolated with a retractable door. One compartment lit with a bright cold house light as the safe compartment while the other made from dark opaque walls and roof as unsafe side. The floor in both compartments made of metal shocking grids except that in unsafe side the floor wired to receive an electric shock of 1mA intensity for 1-second duration.

In this test, each animal kept on the dark side at least 10 min to adapt to the darkened compartment for the first two days. On the third day, the animal again put on the safe side while the retractable door was closed. Then, the light in the safe compartment was turned on, and the retractable door was opened to measure the initial latency (IL). As soon as the animal's tail tip crossed the border line on the safe side and the unsafe side, the retractable door released and an electric shock of 1mA was applied to the animal limbs through the floor grids. After the shock delivery, the animal returned to its cage for the test session. On day 27, the testing trial was done just like the day before, except that no foot shock applied, and the step-through latency (STL) measured. In this test, a 450 seconds cut-off time was established if the animal did not cross the border.

Statistical analysis

Data analysis was performed using SPSS 20 statistical software. Data were analyzed with Kruskal-Wallis non-parametric test. In all statistical analysis, $P<0.05$ was considered significant.

Results

Data expressed in figure 1 demonstrates respective STL of passive avoidance memory in experimental groups. As noted, no statistical differences were found among IL of the experimental groups.

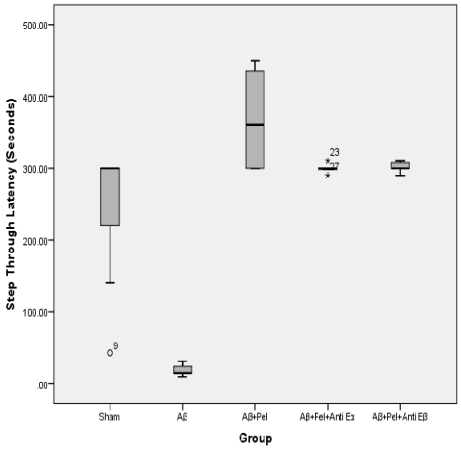

Figure 1. Passive avoidance task illustrates step through latency among the experimental animals

Bilateral CA1microinjectios of saline 2µl (Sham), $A\beta_{25-35}$ 5µg (Aβ) , $A\beta_{25-35}$ + Pelargonidin 10 mg/kg (Pel) orally fed for 3 consecutive pretreatment days (Aβ + Pel), $A\beta_{25-35}$ + Pel orally fed for 3 consecutive pretreatment days + unilateral intra cerebroventricular (i.c.v.) microinjection of α estrogen receptor antagonist (Aβ + Pel + Anti Eα), $A\beta_{25-35}$ + Pel orally fed for 3 consecutive pretreatment days + unilateral i.c.v, microinjection of β estrogen receptor antagonist (Aβ + Pel + Anti Eβ).

However, STL was profoundly lowered in Aβ treated rats compared with sham animals (P=0.002). Three days pretreatment with Pel (10 mg/kg) in Aβ + Pel caused a significant increase in STL (P=0.02) compared with the Aβ group. Aβ + Pel + Anti Eα (P=0.003) and Aβ + Pel + Anti Eβ (P=0.003) groups followed the same track. Compared with Aβ + Pel, STL has not been changed among Aβ + Pel + Anti Eα (P=0.11) and Aβ + Pel + Anti Eβ (P=0.41) groups.

Discussion

In the presented study, we investigated the potential memory-enhancing and the estrogenic receptor role of oral Pel pretreatment in an $A\beta_{25–35}$ rat model of AD. Our findings show that bilateral CA1 hippocampal microinjections of $A\beta_{25–35}$ caused significant memory loss in passive avoidance task paradigm. This finding is in agreement with similar studies in which bilateral CA1 intrahippocampal microinjections of $A\beta_{25-35}$ fragment produced reactive oxygen species followed by oxidative stress insults, neuronal degeneration and cell loss of the pyramidal cells affecting memory loss in rats (3).

In passive avoidance performance, we found that bilateral CA1 hippocampal $A\beta_{25-35}$ microinjections before the training phase of the classical one-trial 24-hour foot shock avoidance application could effectively lengthen STL compared to control animals. This suggested that the substance was negatively affected long-term memory (LTM) while keeping IL untouched in all experimental groups. Pretreatment with Pel in the $A\beta_{25-35}$ group could ameliorate defective LTM without disturbing normal ambulatory scenario even after about one month.

Estrogen and its receptor modulators have shown to have a neuroprotective role in neurodegenerative conditions such as stroke, AD, and Parkinson disease. Although the exact mechanisms of this neuroprotection have not been exactly clarified, some leading possibilities have been addressed in the literature (24).

Coadministration of α and β estrogen antagonists with Pel in the $A\beta_{25-35}$ group could not change LTM in passive avoidance task performance. This stresses on the existence of estrogen derivative neuroprotection on LTM through other available mechanisms than typical estrogen receptors (ERs). ERs are largely spread over some memory related areas of the brain like the hippocampus, frontal cortex and amygdala (25). Estrogens' neuroprotection may mediate mainly via ER-dependent of genomic (nuclear) and non-genomic (extranuclear) and ER-independent pathways. In non-genomic pathway, this may be achieved via G protein-coupled receptor and tyrosine kinase receptor activations plus membrane fluidity alteration, K+ channel opening, and Ca+2 influx. The ER- independent estrogen neuroprotection is mainly attained through free radical scavenging, neuronal excitability and neurotransmission changes (26).

Flavonoids can apply their neuroprotection in neurodegenerative diseases and aging via other different ways among which neuronal anti-inflammatory is a matter of vast studies (27). Pel has shown its own anti-inflammatory effects when used in pro-inflammatory conditions such as macrophage exposure to lipopolysaccharide. The effect is mainly mediated by inducible nitric oxide synthase (iNOS) suppression and nuclear factor-κB (NF-κB), and signal transducer and activator of transcription 1 (STAT-1) inhibition (28-30). Although the studies showing parent anthocyanin anti-inflammatory response in neuronal architecture are rather limited (31), their intestinal metabolites and bacterial bowel derivatives have shown to be effective in neuronal anti-inflammatory conditions (27).

Anthocyanins antihypertensive and endothelial related relaxatory effects have shown in animals under a high cholesterol food protocol (13). This is basically mediated through bilitranslocase, an endothelial plasma membrane flavonoid transporter with vasodilatory properties (30). Therefore, improved blood flow especially in the hippocampal region following Pel use may stimulate neurogenesis and memory function. Moreover, increased dendritic spine density and morphology repair may also improve neuronal connectivity and memory performance (32). This would also be considered as another possible explanation for memory enhancing effects of ER agonists as Pel.

Anthocyanin cellular responses can also be governed through signaling cascades such as phosphoinositide 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK), the indirect mammalian target of rapamycin (mTOR). Cell growth initiation through various regulatory pathways and pro-apoptotic molecules inactivation-like Bad to promote cell survival (18,30,33) are other presumptive ways that Pel may adopt to carry its positive memory effects.

In conclusion, in this study results from passive avoidance test paradigm showed that three days oral Pel pretreatment (10 mg/kg) could reverse $A\beta_{25-35}$ induced memory disturbance via ERs independent pathways. Targeting different pathological mechanisms, Pel would be one of the valuable natural substitutes for estrogen to prevent age-related cognitive changes and memory deficit states like AD. However, more studies should be done to show its exact mechanisms and other beneficial influences to reach this goal.

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