Research Paper: The Ameliorative Impact of *Centella asiatica* on the Working Memory Deficit in Streptozotocin-induced Rat Model of Alzheimer Disease

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ABSTRACT

Introduction: Alzheimer disease (AD) is a complex neurodegenerative disorder with a progressive nature leading to neural damage and cognitive and memory deficit. The present study investigated the neuroprotective effects of *Centella asiatica* (CA) in Streptozotocin (STZ)-induced rat model of memory impairment and neuronal damage.

Methods: The intracerebroventricular infusion of STZ (3 mg/rat) or saline (as the vehicle) was performed on days 1 and 3. CA (150 and 300 mg/kg/d) was administered through oral gavage for 21 days after model induction. We used the Y-maze test to assess the working memory-related performances of animals. Rats were then sacrificed, and their hippocampi were harvested for evaluation of neuronal density in the cornu ammonis (CA1, CA2, CA3) and Dentate Gyrus (DG) regions using stereology technique.

Results: The intracerebroventricular infusion of STZ caused significant working memory impairment demonstrated in the Y-maze apparatus, with a significant decrease in alternative behavior compared to control animals (40.67±2.04 vs 73.00±1.88, P<0.0001). Oral administration of CA (150 and 300 mg/kg each day) for 21 days significantly improved STZ-induced working memory deficit (55.33±3.34 and 57.17±3.81 vs 40.67±2.04, P<0.013, P<0.004, respectively). Furthermore, 21 days of consecutive administration of CA significantly ameliorated STZ-induced neuronal loss in the CA1, CA2, and DG subfields of the hippocampus.

Conclusion: Overall, these data demonstrate that CA increases neuronal density and improves cognitive impairment in the STZ-induced rat model of AD, thereby having promising therapeutic potential for neurodegenerative disorders. Accordingly, further studies are needed to determine the exact molecular mechanism of CA protective effects in brain disorders, particularly AD.

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Highlights

- *Centella asiatica* (CA) improved the STZ-induced working memory deficit.
- CA could prevent hippocampal neural cell loss dose-dependent manner.
- CA improved memory through mitigating neuronal loss in hippocampus.

Plain Language Summary

Memory loss is the first signs of dementia. It is well known that a healthy diet might be as good for your brain as it is for your heart. Numerous traditionally used medicinal herbs could significantly affect key events culminating in dementia and Alzheimer's disease. *Centella asiatica*, commonly known as Gotu Kola or Indian Pennywort, is a tropical, medicinal plant native to Southeast Asian countries. It is one of the becoming popular medicinal plants in the world. *Centella asiatica* (CA) is widely used in different traditional medicine systems for various purposes, such as reducing blood pressure, memory enhancement, and promoting longevity. In the present study, we tested the possible impact of CA leaf and stem extract in an animal model of memory damage. Memory impairment was induced in adult rats by intracerebral infusion of a neurotoxin chemical. Then, the memory-impaired animals were orally treated with 150-300 mg/kg of CA extract for 21 days. Finally, we tested their working memory by placing them in a Y-maze apparatus. Furthermore, their most involved brain part (hippocampus) was dissected, and its cell density was evaluated. Our findings exhibited that CA treatment considerably improved rats' memory performance, indicating by enhancing working memory score in the Y-maze task. In addition, CA treatment significantly prevented neuronal cell loss in the hippocampus of memory-impaired rats. This study shows that CA has beneficial effects on memory and cognitive function.

1. Introduction

Alzheimer Disease (AD) is a progressive and age-related amyloid pathology associated with neural loss as well as learning and memory deficits (Morgan et al., 2000). Intracerebroventricular (ICV) infusion of the low dose of Streptozotocin (STZ) is widely used to induce sporadic AD (sAD) model (Rani, Deshmukh, Jaswal, Kumar, & Bariwal, 2016). The underlying mechanism is loading massive amyloid that commences inflammatory processes (Bellenger et al., 2011) and causes neural damage and progressive cognitive decline (Hayate Javed et al., 2011; Shi, Zhang, Li, & Hölscher, 2017; Shingo, Kanabayashi, Kito, & Murase, 2013).

Memory is a process through which information is stored and retrieved (Kandel et al., 2000). Several brain structures are responsible for learning and memory formation; among them, the hippocampus has a prominent role (Hall, 2015; Robbins, Ersche, & Everitt, 2008; Ying et al., 2002). Accordingly, hippocampal lesions are frequently associated with memory loss and cognition impairment (Broadbent, Squire, & Clark, 2004). Also, many investigations have mentioned the inflammatory cell death alongside a significant synaptic loss and reduced neural density in the hippocampus of human subjects with AD or animal models of cognition and memory impairments (Bancher, Braak, Fischer, & Jellinger, 1993; Terry et al., 1991).

In recent decades, herbal medicine has gained prominence in the studies of human brain disorders. Many investigations addressed the preventive, protective, or ameliorative effects of herbal extracts in animal models of neurodegenerative diseases, including AD (Dhanasekaran et al., 2009; Soumyanath et al., 2012; Veerendra Kumar & Gupta, 2003). *Centella asiatica* (CA) belongs to the family of Apiaceae (Umbelliferae) (Kapoor, 2017) and shows several beneficial effects on the Central Nervous System (CNS), such as inducing mental calmness, reducing anxiety, and enhancing intelligence and memory (Kumar & Gupta, 2002; Seevaratnam, Banumathi, Premalatha, Sundaram, & Arumugam, 2012). In ancient India, CA has considered a treatment for dementia (Shinomol & Bharath, 2011). It has been shown that CA extract decreases oxidative stress markers (Gray, Harris, Quinn, & Soumyanath, 2016; Haleagrahara & Ponnusamy, 2010; Jayashree, Muraleedhara, Sudarslal, & Jacob, 2003). Moreover, this herbal extract has been demonstrated to reduce amyloid-beta levels in the hippocampus (Dhanasekaran et al., 2009).

The present study aimed to explore the effect of chronic oral administration of CA aqueous extract against STZ-induced cognitive impairment and reduced neural density in the cornu ammonis (CA1, CA2, CA3) and Dentate Gyrus (DG) subfields of the hippocampus.

2. Methods

Animals

Adult male Wistar rats (weighing 200-250 g) were obtained from the Research Centre of Experimental Medicine, Birjand University of Medical Sciences. The rats were housed in polypropylene cages (3 per cage) under controlled condition (12 h light/ 12 h dark cycle, lights on 07:00) and temperature (21±2°C) with free access to food and water except for limited periods of experiments. Behavioral tests were performed during the light cycle between 8:00 to 14:00. Six animals were used in each experimental group, and each animal was tested once. All experiments and animal care were conducted according to guidelines of the Ethics Committee of Birjand University of Medical Sciences (Ethical code: IR.BUMS.REC.1387.298).

Chemicals and agents

Streptozotocin (STZ) was purchased (Sigma-Aldrich, St. Louis, MO, USA) and dissolved in sterile and cold saline. The CA lyophilized powder was bought (TST Plant Medicines Co, Tehran, Iran) and dissolved in distilled water to obtain desired concentrations. Ketamine (Bremer Pharma GMBH, Bremerhaven, Germany) and xylazine (Alfasan Chemical Co., Woerden, Holland) were used for animal anesthesia. Toluidine blue was purchased from Merck Company (Germany). Other chemicals were bought from Merck or Sigma-Aldrich.

Surgery and drug treatment

For surgery, animals were anesthetized with Intraperitoneal (IP) injection of ketamine (80 mg/kg) and xylazine (15 mg/kg) and were placed in a stereotaxic device (Stoelting, Wood Dale, IL, USA). Ketamine and xylazine doses were chosen according to the previous research (Adeli, Zahmatkesh, Tavoosidana, Karimian, & Hassanzadeh, 2017; BonakdarYazdi et al., 2017; Wang, Wang, Cheng, & Che, 2016). The 22-gauge guide cannula was fixed into the lateral ventricles according to the atlas of Paxinos and Watson (AP: 0.8 mm; ML: 1.8 mm; DV: 3.6 mm) (Zilles, 2012). ICV infusion of STZ (3 mg, 1.5 mg/5 µL/side) was infused on days 1 and 3 through an injection needle (27 G) connected to a 50-µL Hamilton syringe (Hamilton, Reno, Nevada). Then after each injection, the needle was left for a further 30 s to facilitate the release of the drug solution. One week after recovery from stereotaxic surgery, normal rats were used for working memory assessment. The effective doses of CA were selected according to previous studies (Chanana & Kumar, 2017; Sari et al., 2014a; Wanasuntronwong, Tantisira, Tantisira, & Watanabe, 2012). CA (150 and 300 mg/kg/d) was administered with oral gavage for 21 days after model induction.

Experimental groups

In this experiment, 30 rats were randomly divided into 5 groups (n=6): 1) control group without surgery or drug intervention; 2) vehicle group received bilateral ICV infusion of saline; 3) STZ group received bilateral ICV infusion of STZ (3 mg, 1.5 mg/5 µL/side, on days 1 & 3); 4) STZ+CA150 animals received bilateral ICV infusion of STZ (3 mg, 1.5mg/5µL/side) on days 1 and 3 and oral gavage of CA (150 mg/kg, daily) from day 14 to day 35; and 5) STZ+CA300 animals received bilateral ICV infusion of STZ (3 mg, 1.5 mg/5 µL/side) on days 1 and 3 and oral gavage of CA (300 mg/kg, daily) from day 14 to day 35.

Y-maze test

Working memory of rats was evaluated using the Y-maze apparatus on day 14 for the STZ group and day 35 for STZ+CA groups. Y-maze test was done according to previous studies (Aminyavari, Zahmatkesh, Farahmandfar, Khodagholi, Dargahi, & Zarrindast, 2019; Hughes, 2004). This device comprises three symmetrical arms (30 × 20 × 10 cm) named A, B, and C. The animals were placed at the end of one of the three arms and were allowed to explore. The procedure was conducted in an 8-min session with no interval. The alternative behavior, the overlapping triplet sets of consecutive entries into three different arms (A, B, and C) of the Y-maze apparatus of rats, was recorded. For instance, if the recorded sequence of arms entries is ABCACBBAC, we could consider four sets of correct alternative movement: 1) ABCACBBAC , 2) ABCACBBAC , 3) ABCACBBAC , and 4) ABCACBBA. Animals' behavior was recorded by a camera placed above the Y-maze device. Surfaces of the apparatus were cleaned with alcohol between sessions. The percentage of alternative behavior was determined using the following calculation:

$$\frac{\textit{Entries into three different arms consecutively}}{\textit{Number of total arm entries-2}} \times 100$$

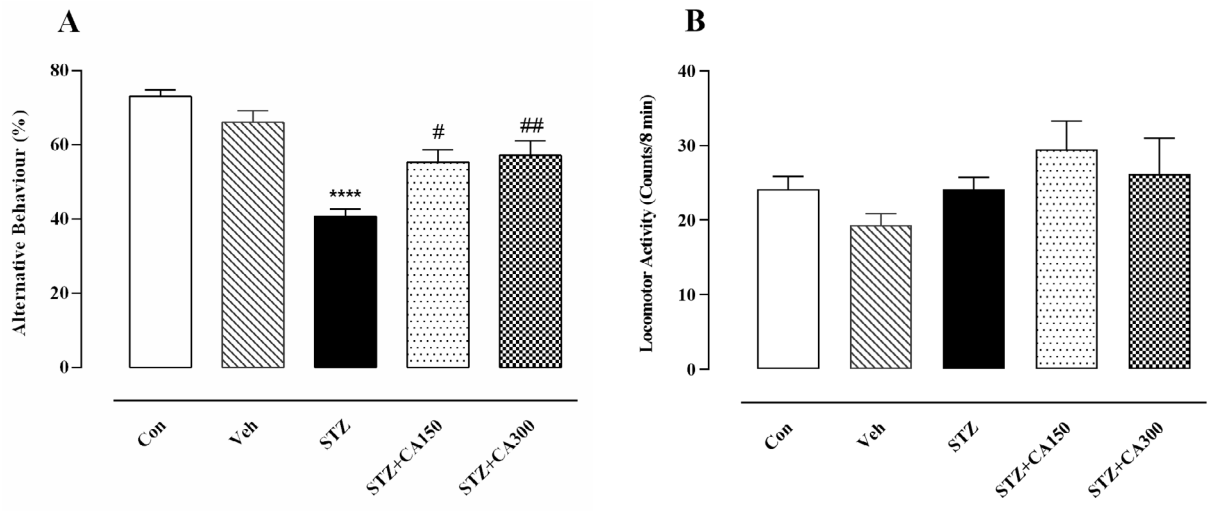

Figure 1. *Centella asiatica* (CA) improved Streptozotocin (STZ)-induced working memory deficits

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Animals received ICV infusion of saline/STZ alone or combined with CA (150 and 300 mg/kg). CA significantly increased the alternative behavior at doses of 150 and 300 mg/kg (A). No significant difference was observed between the groups regarding the total arms entries (B).

****P<0.0001 compared to the Control group. #P<0.01 and ##P<0.004 compared to the STZ -treated rats. Values are expressed as mean±SEM (n=6 in each group)

Tissue preparation

After completing the behavioral test, the animals were anesthetized with ketamine and xylazine mixture and then perfused with Phosphate-Buffered Saline (PBS) followed by 4% paraformaldehyde. The animal's brain was harvested and subsequently immersed in a fixative solution (4% paraformaldehyde) for 48 h at 4 °C. After the dehydration using different degrees of alcohol, the samples were embedded in paraffin block and sectioned transversely (7 µm thick) using a microtome device (Leitz, Italy). The sections were then stained by toluidine blue for counting the number of neurons in different areas of the hippocampus. The sections were deparaffinized in xylene and rehydrated through a descending ethanol series. After that, the slides were rinsed in a staining dish containing 1% toluidine blue in 1% sodium borate solution (1 g of toluidine blue and 1 g of sodium borate in 100 mL distilled water) and allowed to react overnight at room temperature. The sections were then dehydrated through methanol (two times), cleared in xylene, and mounted in Entellan (Hami, Hosseini, Shahi, Lotfi, Talebi, & Afshar, 2015).

Stereological analysis

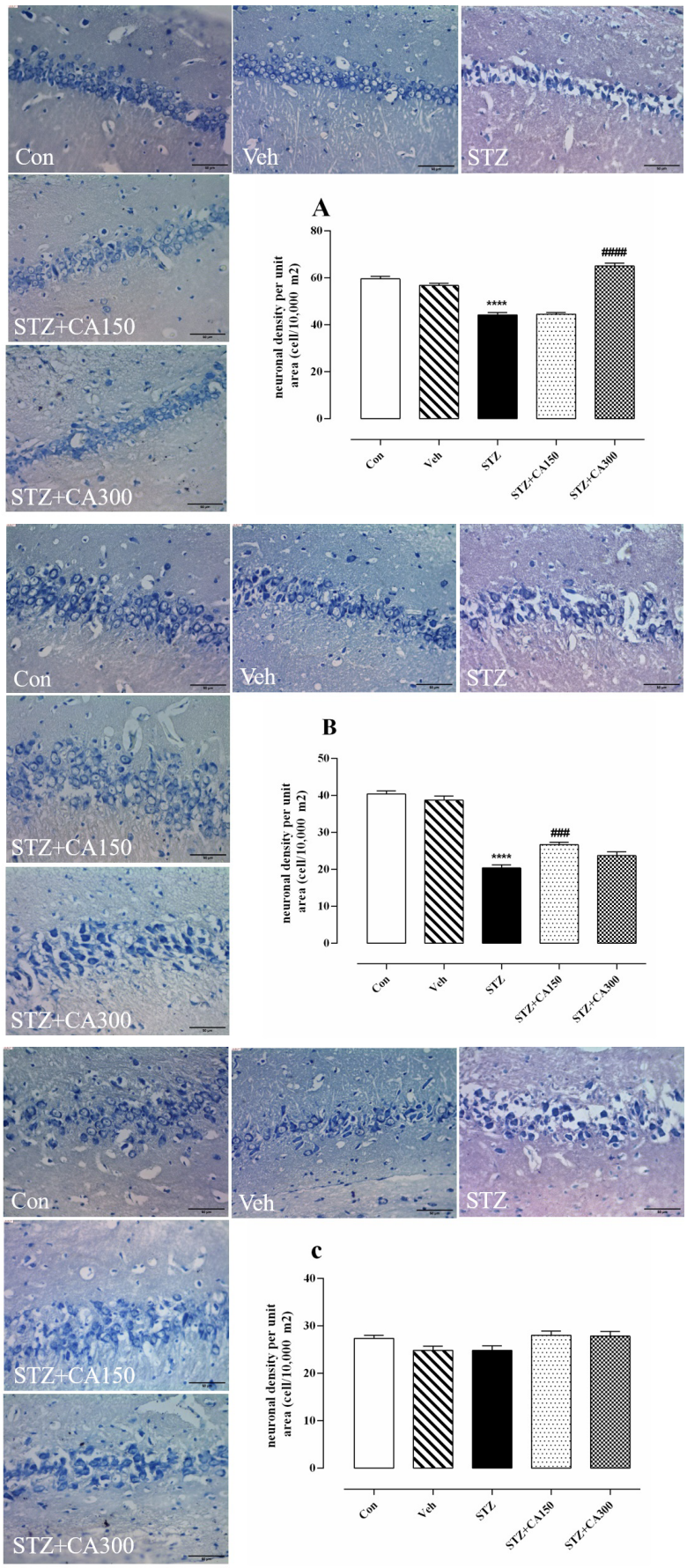
The number of neurons in different hippocampus regions (CA1, CA2, CA3, and DG) were counted using the optical dissector method (Gundersen, 1988). For counting, 8-10 sections in each hippocampus region were randomly selected. The sections were then observed under a light microscope (UPlan FI, Japan), connected to a computer by a high-resolution camera (BX51, Japan). Rectangular grids were placed on the examination area, and the number of neurons was calculated under 40× objectives. Finally, the neuronal density was calculated in different regions of the hippocampus by the following equation:

$$NA = \frac{\sum Q}{a/f.\sum p}$$

In this formula, ∑Q is the summation of counted neurons that appeared in sections, "a/f" is the area associated with each frame (10000 µm^2), and "∑P" is the sum of frames associated points hitting the reference.

Statistical analysis

These analyses were performed using the GraphPad Prism V.6. One-way Analysis of Variance (ANOVA) followed by Tukey post hoc test was used for multiple comparisons of data gathered from the Y-maze device and the number of neurons in different hippocampus regions. P<0.05 was considered the level of significance. All results were presented as Mean±SEM.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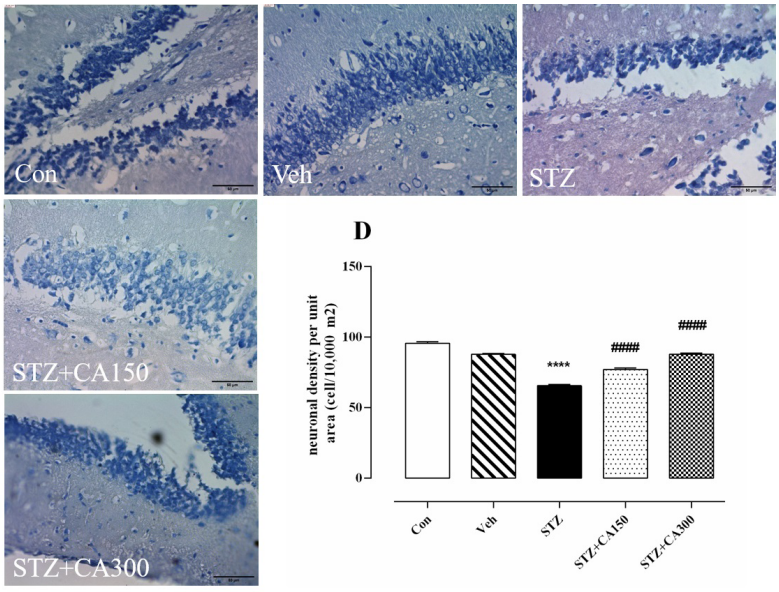

Figure 2. Neuroprotective effect of *Centella asiatica* (CA) against Streptozotocin (STZ)-induced neurotoxicity NEUROSCIENCE

Alteration of neuronal cell density in various subfields of the hippocampus. A: CA1, B: CA2, C: CA3, and D: DG.

*P<0.001 compared to the control group. #P<0.001 compared to STZ treated rats. Values are expressed as Mean±SEM (n=6 in each group).

3. Results

Effect of CA on memory performance in Y-maze

Behavioral findings showed a significant effect of CA on the working memory of rats in the Y-maze apparatus ($F_{4, 25}$=17.05, P=0.0001). Bilateral ICV infusion of STZ diminished working memory, demonstrated by a significant decrease in alternative behavior than the control group (40.67±2.04 vs 73.00±1.88, P=0.001). Oral administration of CA for 21 days significantly improved STZ- induced memory dysfunction at doses of 150 mg/kg (55.33±3.43 vs 40.67±2.04, P=0.013) and 300 mg/kg (57.17±3.81 vs 40.67±2.04, P=0.004) (Figure 1A). Hence, CA showed an ameliorative effect in the STZ-induced rat model of AD. It is worth mentioning that no significant difference was observed in the total arm entries ($F_{4, 25}$=1.51, P=0.22; Figure 1B) among groups, indicating that alterations in the behavior of animals were not related to musculoskeletal or motor dysfunction.

Effect of CA on neuronal density in different areas of the hippocampus

Histological analysis showed that CA had a significant effect on neural density in the CA1, CA2, and DG subfields of the hippocampus (CA1: $F_{4, 25}$=81.35, P<0.001; CA2: $F_{4, 25}$=92.30, P<0.001; DG: $F_{4, 25}$=159.2, P<0.001). Neural density was significantly reduced in the CA1, CA2, and DG areas of the hippocampus of animals that received ICV infusion of STZ compared to the control group (CA1: 44.17±0.94 vs 59.50±1.14, P<0.001; CA2: 20.33±0.88 vs 40.33±0.84, P<0.001; DG: 65.50±0.84 vs 95.67±1.08, P<0.001). Oral administration of CA for 21 days increased neuronal density in STZ-treated animals in CA1 (300 mg/kg: 65.00±1.29 vs 44.17±0.94, P<0.001), CA2 (150 mg/kg: 26.67±0.66 vs 20.33±0.88, P=0.0006) and DG (150 mg/kg: 77.00±1.06 vs 65.50±0.84, P<0.001; 300 mg/kg: 87.83±0.87 vs. 65.50±0.84, P<0.001) compared to the control group (Figure 2A, B, and D). No significant difference was observed in the number of neurons in the CA3 subfield of the hippocampus among groups (P>0.05, Figure 2C).

4. Discussion

The present study examined the ameliorative effect of CA extract in the animal model of sporadic dementia of Alzheimer type developed by ICV infusion of STZ. Memory impairment is the most important symptom of AD; hence we engaged the Y-maze device to assess working memory deficit during the model induction. Bilateral ICV infusion of STZ (3 mg/kg) could impair the working memory of rats after 14 days, as indicated by a significant reduction in alternative behavior compared to control animals (Figure 1A). Histological assessments also showed a remarkable decrease in neuronal density

in different hippocampus areas, which confirmed the behavioral outcome (Figure 2).

Based on a wealth of evidence, the hippocampus plays an essential role in different types of learning and memory. In this regard, hippocampal lesions contribute to significant memory impairment in human subjects or animal models (De Haan, Mishkin, Baldeweg, & Vargha-Khadem, 2006). In line with our results, previous studies have demonstrated long-term and progressive memory impairment following ICV infusion of STZ, which is similar to the sporadic type of AD (Lannert & Hoyer, 1998; Sharma & Gupta, 2001; Shi et al., 2017). Moreover, STZ produces a significant neuronal loss in the brain areas, including the hippocampus (Dhull, D. K., Jindal, Dhull, Aggarwal, Bhateja, & Padi, 2012; Song et al., 2014; Veerendra Kumar & Gupta, 2003). STZ enhances glucose levels in the rat's brain and, in turn, improves the conversion of amyloid precursor protein to amyloid-beta, which commences inflammatory processes (Chu & Qian, 2005). These events decrease insulin expression and increase oxidative stress and the production of Reactive Oxygen Species (ROS) in the hippocampus, leading to neural apoptotic death and learning and memory deficit (Ahmed et al., 2013; bAx; H Javed et al., 2012). Accordingly, ICV infusion of STZ could induce a proper animal model of sAD. Several studies demonstrate that the memory impairment following ICV infusions of STZ starts at day 14 and lasts more than 35 days (Afshar, Shahidi, Rohani, Komaki, & Asl, 2018; Agrawal, Tyagi, Shukla, & Nath, 2009; Chen et al., 2012). Agrawal et al. demonstrated the STZ-induced spatial memory impairment and oxidative stress induction in rat brain after 14 days (Agrawal et al., 2009). Furthermore, Afshar et al. reported the novel object recognition and passive avoidance learning impairment and a significant decrease in hippocampal BDNF levels 35 days after ICV STZ administration (Afshar et al., 2018).

In addition, our results showed that oral administration of CA for 21 days after the development of the AD model improved working memory, as indicated by increased alternative behavior of rats (Figure 1A). We also observed that CA administration (150 and 300 mg/kg/d) increased neural density in the CA1, CA2, and DG subfields of the hippocampus in STZ-treated animals (Figure 2A, B, and D). Thereby, CA significantly ameliorates memory deficit and hippocampal damage in STZ-induced animal model of sAD.

Flavonoid enriched plants exert powerful action on the memory of mammals. These compounds maintain the normal functioning of neurons and even limit age-related neurodegeneration (Vauzour, 2014). CA contains flavonoid compounds making it a potential herbal remedy for AD (Zainol, Abd-Hamid, Yusof, & Muse, 2003). Moreover, asiatic acid, a triterpenoid, is present in CA extract and is a neuroprotective agent (Rather, Justin-Thenmozhi, Manivasagam, Saravanababu, Guillemin, & Essa, 2019).

Many studies have shown the safety borders of CA (Chauhan & Singh, 2012; Chivapat, Chavalittumrong, & Tantisira, 2011; Roopesh, Salomi, Nagarjuna, & Reddy, 2011). The CA LD50 value was determined to be more than 10 g/kg suggesting a comparatively high margin of safety (Chivapat et al., 2011). CA functions as an antioxidant agent and free radical scavenger that prevent lipid peroxidation and DNA damage (Dhanasekaran et al., 2009; Rahman, Sayeed, Haque, Hassan, & Islam, 2012; Veerendra Kumar & Gupta, 2003). Brain oxidative damage and mitochondrial dysfunction, as well as programmed neurons death, occur in the STZ-induced animal model of sAD, which is responsible for cognitive and memory impairment (Saxena, Patro, & Nath, 2011; Sharma & Gupta, 2001). Interestingly, CA improves mitochondrial enzymes activity and reduces caspase-3 and Bax/bcl-2 levels (anti-apoptotic factors) in the hippocampus (Prakash & Kumar, 2013). Accordingly, CA can inhibit apoptotic neural death in the hippocampus of AD animal models via affecting AKT/GSK3β signaling pathway (Rather et al., 2019).

Moreover, CA enhances phosphorylation of Cyclic AMP Response Element-Binding protein (CREB) as an essential transcription factor through ERK/RSK signaling pathway (Xu, Cao, Khan, & Luo, 2008). CREB regulates the expression of critical genes, including Brain-Derived Neurotrophic Factor (BDNF) and cholinergic markers (Xu et al., 2008). Nerve growth factors are crucial for hippocampal synaptic plasticity and nerve growth, as well as learning and memory formation (Hsiao, Hung, Chen, & Gean, 2014). A significant reduction in BDNF has been observed in the hippocampus of the STZ-induced model of sAD (Afshar et al., 2018). Strikingly, CA can improve BDNF level in rats' hippocampus, which makes it an important candidate to relieve AD symptoms (Handayani, Yolanda, & Kodariah, 2018). Cholinergic hypofunction is significantly involved in memory impairment induced by ICV infusion of STZ (Agrawal et al., 2009). Due to the effect of CA on CREB activity, it is expected to enhance the transcription of cholinergic markers, choline acetyltransferase, and vesicular acetylcholine transporter. On the other hand, CA inhibits inducible nitric oxide synthase activity, thereby enhancing cholinergic neurotransmis-

sion, which is critical for learning and memory formation (Sari et al., 2014b).

Furthermore, CA enhances dendritic arborization and synaptogenesis by modulating ERK1/2 and Akt signaling pathways (Gray et al., 2018). It is suggested that asiatic acid enhances doublecortin and NOTCH1 protein levels in the hippocampus, which promote hippocampal neurogenesis (Sirichoat et al., 2015). Strikingly, it has been demonstrated that CA increases synaptic markers in the hippocampus and frontal cortex through augmentation of the cAMP/PKA signaling pathway (Gray et al., 2016). In addition, CA improves synaptic trafficking by increasing the expression of the AMPAR GluA1 subunit in the hippocampus (Wong et al., 2019).

5. Conclusion

Taken together, we showed the ameliorative impact of CA on STZ-induced working memory impairment and hippocampal neural damage. The detection of the exact molecular mechanism of the effectiveness of CA requires further examination.

Ethical Considerations

Compliance with ethical guidelines

All experiments and animal care were conducted according to guidelines of the Ethics Committee of Birjand University of Medical Sciences (Ethical code: IR.BUMS.REC.1387.298).

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Authors' contributions

Conceptualization and Supervision: Mohammad Reza Saebipour and Mohammadmehdi Hassanzadeh-Taheri; Methodology: Mohsen Foadoddini and Samaneh Aminyavari; Investigation: Razyeh Sahraei and Mehran Hosseini; Data Analysis: Samaneh Aminyavari; Writing - original draft: Samaneh Aminyavari and Mehran Hosseini.

Conflict of interest

The authors declared no conflict of interest.

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