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Research Paper

Neuroprotective effects of empagliflozin against scopolamine-induced memory impairment and oxidative stress in rats

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ABSTRACT

Alzheimer's disease (AD) is one of the most common age-related neurodegenerative disorders. The main medicinal theory for the management of AD belongs to the acetyl-cholinesterase-inhibition pathway and NMDA antagonism. Recent investigation proposed memory improvement by sodium-glucose co-transporter 2 (SGLT2) inhibitors which indicated to improve glycemic control in adults with type 2 diabetes mellitus. According to the lack of sufficient evidence about the efficacy of empagliflozin (EMPA) for memory improvement, in comparison with donepezil (DON), the present research was carried out in order to investigate this hypothesis towards scopolamine-induced neurotoxicity on experimental male Wistar rats. The animals divided into two sets, each included 4 groups: The first set of Healthy animals [Control, EMPA (4 or 10 mg/kg), DON (1 mg/kg)]. The second set of rat Alzheimer model, which received 2 mg/kg Scopolamine by intraperitoneal route for 10 days followed by other treatments [AD, AD+ EMPA (4 or 10 mg/kg) and AD+DON]. Normal rats and AD rats, with each group receiving different substances for 8 consecutive days and 24 h after the accomplishment of the drug administrations, the memory functions assessed through Morris water maze (MWM) paradigm. This task was followed by decapitation of rats in order to evaluate the biochemical oxidative stress parameters in brain tissue. Our data indicated that EMPA significantly improved animals' performance in the behavioral test with a significant decrease in oxidative stress and antioxidant imbalance. In addition, EMPA (4 mg/kg) significantly reduced both cellular malondialdehyde and protein carbonyl content while conversely increased the total reduced glutathione content. Besides, the levels of total as well as endogenous antioxidants in the ferric reducing antioxidant power assay reported to be augmented. It seems that EMPA significantly improved both cellular biochemical aspects and memory performance in animal models in accordance with histopathological assessments. Conclusively, 4 mg/kg EMPA demonstrated better results in all aspects that were evaluated during this research.

1. Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disease is represented by the continuous decline of memory and impaired executive functions and transitions in behavior and personality. AD is the sixth leading cause of death in the United States and the fifth leading cause of death for people over age 65 (Kandimalla et al., 2017). Initial symptoms of AD are changes in thinking or unaware behavior, inability

Abbreviations: AD, Alzheimer's disease; Aβ, Amyloid β; SCOP, scopolamine; EMPA, empagliflozin; LPO, Lipid peroxidation; PCO, Protein carbonyl; GSH, Glutathione; FRAP, Ferric reducing ability of plasma; TPTZ, [2,4,6-Tri2-pyridyl-s-triazine]; DTNB, [5,5′-Dithiobis 2-nitrobenzoic acid]; AChE, Acetylcholinesterase; MDA, Malondialdehyde.

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to memorize new information, and dysfunctional changes in language and speech (Tarawneh and Holtzman, 2012). Meanwhile, neuropathological features include the formation of senile plaques from amyloid beta peptide, neurofibrillary tangles, increased phosphorylated tau, and neuronal and synaptic loss (Serrano-Pozo et al., 2011). On the other hand, while the exact mechanism for the onset of dementia in patients with Diabetes Mellitus is not yet fully understood, potential causes seem to be disruption of the blood–brain barrier due to hyperglycemia and impaired insulin signaling (Steen et al., 2005; Rom et al., 2020). Moreover, according to the fact that SGLT-2 inhibitors are lipid-soluble and capable of penetrating the blood–brain barrier, they can affect the SGLT-1/SGLT-2 cotransporter, which is widely expressed in the brain and has been implicated in learning processes and long-term memory, via reducing brain inflammation as well as apoptosis (Mone et al., 2022; Youn et al., 2024). Furthermore, findings of a meta-analysis indicated a positive effect of SGLT-2 inhibitor use on cognitive function score improvement, particularly among populations with mild cognitive impairment or dementia (Youn et al., 2024).

However, defects in insulin receptor sensitivity, oxidative stress, neuroinflammation, intracellular signaling and mitochondrial metabolism have already been implicated as factors in the occurrence of Alzheimer's disease (Rizzo et al., 2022).

The findings of studies in recent years show that the brain of AD patients may be a special form of the brain of DM patients (type 3 diabetes), therefore, the role of beta amyloid and tau protein in the peripheral nervous system along with related organs and also in the induction of insulin resistance is strong so significant results have been obtained the relationships and common mechanisms between them (Sandhir and Gupta, 2015). The regarded effect was proven with application of insulin sensitizer in rodent model, which reversed the acetylcholine homeostasis and cognitive function. The formation of Aβ plagues disturbs the synthesis of brain intrinsic insulin, and hinders the transportation of GLUT4 protein into hippocampal cellular membrane, which leads to malfunction in insulin-like growth factor (IGF) and leptin production. As a result, a lowered neural cell potency in synaptogenesis and energy production as the dominant AD biological manifestation is occurred (Rad et al., 2018).

It was suggested that risk of dementia and AD increased in type 2 diabetes (T2DM), which was further supported by clinical and epidemiological studies. Patients with diabetes have 2 fold higher risk of AD in compare to non-diabetic patients (Sandhir and Gupta, 2015). Studies have shown that there are many common pathophysiological mechanisms between AD and T2DM, associated with insulin resistance, such as oxidative stress, mitochondrial dysfunction, insulin signaling disorder, neuro inflammation, advanced glycosylation end products (AGEs) and metabolic syndrome (Michailidis et al., 2022).

One of the newest class of oral anti-diabetic agents are sodium-glucose co-transporter 2 inhibitors (SGLT2i), which are approved for treatment of diabetes mellitus (Nauck, 2014). SGLT2 inhibitors, have glucose-lowering effects and this affects metabolism in different ways (Lingli and Wenfang, 2022). SGLT2 inhibitors mediates glucose reabsorption in the early proximal tubule in kidney. Therefore, treatment with this drugs increased urinary glucose excretion. This has been observed in type 2 diabetic patients and multiple animal models of T2DM. This led to a reduction in blood glucose and in chronic treatment, a reduction in HbA1c in animal models and patients (Michel et al., 2015). It has been showed that Empagliflozin, a selective sodium-glucose co-transporter-2 inhibitor, has additional non-glycemic benefits, including neuroprotection (Motawi et al., 2022).

Recently a new type of diabetes under name of diabetes type 3 has been introduced which accompanies a reduction in brain functioning and learning (Kandimalla et al., 2017). The recent data suggested the neuroprotective effect of dapagliflozin in high-fat diet-induced obesity rat models through improving memory and spatial learning ability (Motawi et al., 2022). A limited data have shown that AD is one of the causes of dementia that exclusively develops in the brain due to insulin resistance and growth factor deficiency similar to what happens in other parts of the body with diabetes types 1 and 2 (Alafnan, 2020; Wiciński et al., 2020; Pawlos et al., 2021; Stanciu et al., 2021). In fact, diabetes type 3, defines people who are affected by diabetes type 2 and dementia which called AD which needs to be categorized as a type of diabetes under name of diabetes type 3.

Scopolamine is a muscarinic receptor antagonist that blocks cholinergic neurotransmission and concomitant appearance of transient cognitive amnesia and electrophysiological changes, leading to memory impairment in Rodents (Yadang et al., 2020; Anoush et al., 2022a; Hosseini et al., 2022). Recent studies have reported other interfering mechanisms such as increasing the accumulation of reactive oxygen species that induces oxidative stress leading to memory impairment (Skalicka-Wozniak et al., 2018; Yadang et al., 2020). Therefore, based on limited data on protective effect of SGLT2 Inhibitors, we decided to investigate neuroprotective effect of Empagliflozin (EMPA) against scopolamine-induced memory impairment in rats using multi-parametric assay including behavioral test by Morris water maze (MWM) test, oxidative stress biomarkers and histopathological assessment in brain.

2. Material and methods

2.1. Animals

A total number of 80 male Wistar rats (Pasteur Institute, Tehran, Iran) weighing approximately 180–200 g were housed in plastic cages in an environmentally controlled room (12-h dark/12-h light cycle, 23 ± 1°C) and had free access to food and water. All the protocols were approved by the Animal Ethics Committee of Zanjan University of Medical Sciences (IR.ZUMS.REC. 1399.338 (prior to the experiments, and animals were treated in accordance with the ARRIVE Guidelines for handling the laboratory animals.

2.2. Experimental design

The scopolamine model for AD-like induction in rat has been performed base on previous pilot studies and published papers (Hosseini et al., 2022). The experiment involved two sets of animals: normal rats and AD rats, with each group consisting of 10 rats, receiving different substances for 8 consecutive days as described below: Healthy groups: (1) Control group which only received normal saline; (2) EMPA (4 mg/kg) group which received 4 mg/kg of empagliflozin by gavage for 8 days in normal rats; (3) EMPA(10 mg/kg) group received 10 mg/kg of empagliflozin by gavage for 8 days in normal rats; (4) DON group which received 1 mg/kg of Donepezil by gavage for 8 days in normal rats and Alzheimer animals (AD) which all received either scopolamine or scopolamine with other treatments: (1) AD group which received 2 mg/kg Scopolamine by intraperitoneal route for 10 days; (2,3) AD+ EMPA(4 mg/kg) rats which received 2 mg/kg IP scopolamine and then received 4 or 10 mg/kg of Empagliflozin by gavage for 8 days; and (4) AD+ DON rats which received 2 mg/kg IP scopolamine and then received 1 mg/kg of Donepezil by gavage for 8 days in normal rats. Twenty-four hours after the accomplishment of the drug administrations, the memory functions assessed through Morris water maze (MWM) paradigm (four days of training followed by one day of probe trial). Immediately after the behavioral tests were fulfilled (following probe test), animals were euthanized with ketamine and xylazine (80 and 8 mg/kg) and whole brain tissue was dissected out on ice after decapitation, soaked in the liquid nitrogen and kept at −80°C until to assess biochemical parameters such as total antioxidant capacity (FRAP) level, non-enzymatic antioxidant Glutathione (GSH) level, protein carbonylation (PCO) amount and lipid peroxidation (MDA)level. Finally, for assessment of histological analysis in brain, the collected tissue samples were fixed in 10 % formalin and stored (Fig. 1).

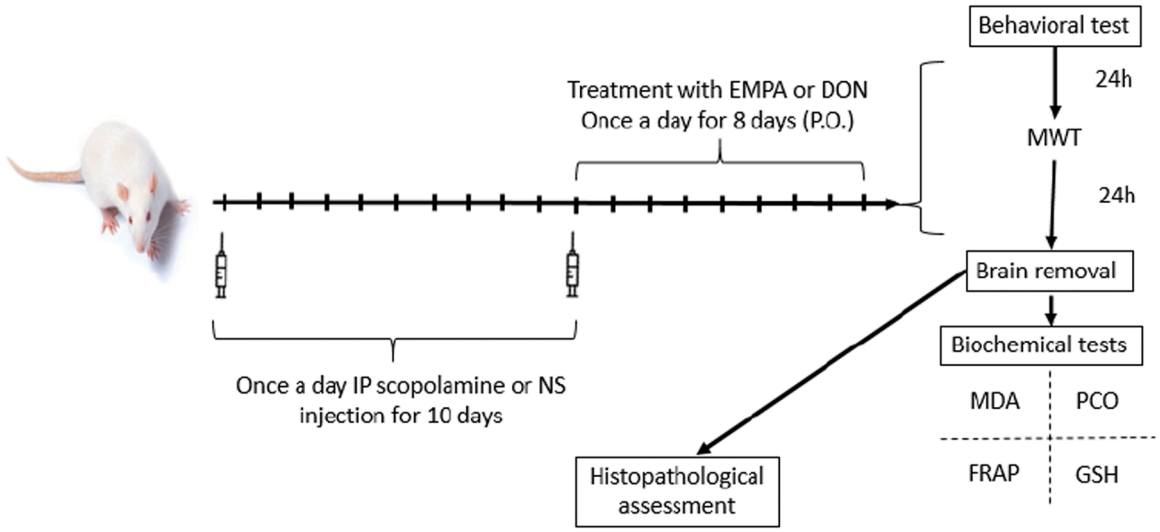

Fig. 1. The timeline of AD like disorder induction procedure, treatment, behavioral and molecular assessments.

2.3. Water maze test

Morris Water Maze (MWM) is an approved behavioral test for assessment of the spatial learning memory ability. During each run, animals were gently floated in circular dark pool from the exact point randomly determined by the computer. The escape platform was submerged under the surface of water. On training days, rats were trained for 4 consecutive days to find the place of the platform. 60 seconds countdown was selected for each animal to swim freely in the pool. In case of finding the platform before 60 seconds, the trial was ended by the software. At the end of each turn, the animals were dried and were transformed into a warm cage. On the 5th day, the platform was removed from the pool. Animals were allowed to swim for 60 seconds. The swimming path patterns and time spent in different quadrants, especially the target quadrant, were counted as criteria for spatial memory assessment (Kandimalla et al., 2017; Berahmand et al., 2020).

2.4. Tissue preparation for biochemical assays

The hole frozen tissue of brain collected samples were separately grinded using tissue grinder. Potassium Chloride solution was also added, and created tissue homogenate was utilized for further biochemical tests.

2.5. Reduced Glutathione (GSH) assay

Glutathione, one of the most important non-enzymatic antioxidant plays a key role in various cellular processes (Roušar et al., 2012). The spectrophotometric method was used based on oxidation of DTNB or Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] to yellow chromophore TNB (5-thio-2-nitrobenzoic acid) at 412 nm (Alisik et al., 2019). Briefly, 50 mg of tissue was mixed and homogenate in 1.5 mL EDTA (0.02 M) and then 0.75 mL of TCA (10 %W/V) was added to suspension. The samples were centrifuged at 3500 rpm for 15 min. The tubes were incubated at room temperature for 15 min. Finally, to 0.5 mL of supernatant, 1.25 mL of tris buffer and 0.25 mL DTNB (0.8 mg/mL; pH=8.9) was added. The absorbance was read at a wavelength of 412 nm following 15 min of incubation at room temperature (Jafari et al., 2022; Anoush et al., 2023).

2.6. FRAP assay

FRAP assay or total antioxidant power assay in brain was determined by TPTZ [2,4,6-Tri(2-pyridyl)-s-triazine] reagent based on reducing ferric (Fe III) to the ferrous (Fe II) form in the sample. Finally, the intensity blue color was measured by spectrophotometric assay at 593 nm (Benzie and Strain, 1996; Bijani et al., 2022). Briefly, 100 mg of brain was homogenate in 1 mL TCA 6 %, then samples were centrifuged at 9000 rpm for 10 min and 0.5 mL of supernatant was mixed by 1.5 mL of freshly prepared FRAP solution [Sodium acetate 300 mM, $FeCl_3$ 20 mM and TPTZ 10 mM (10:1:1 V/V/V)] and the absorbance was read at 593 nm(Bijani et al., 2022).

2.7. Protein carbonyl (PCO) assay

Oxidation of amino acid residues following exposure with high amount of reactive oxygen (ROS) level in pathological conditions caused to protein carbonyls formation in cell (Chevion et al., 2000) based reaction with 2,4-Dinitrophenylhydrazine (DNPH) at 365 nm (Reznick and Packer, 1994). Concisely, 100 mg of tissue was homogenate in 1 mL of tris buffer and then 0.2 mL of the homogenized sample was added to the 0.5 mL of 20 % (w/v) TCA and samples were incubated at 4°C for 15 min. The resulting mixture was centrifuged at 6500 rpm for 10 min. Then, 0.5 mL NaOH (0.1 %) and 0.5 mL DNPH (0.2 %) was added to precipitates. The tubes were left in dark for 1 hr at room temperature. After incubation, 0.5 mL of TCA (20 %) was added and again the mixture was centrifuged at 6500 rpm for 10 minutes. Samples were dissolved in 0.2 mL of guanidine hydrochloride (6 M), the mixture was centrifuged at 5000 rpm for 5 minutes. Ultimately carbonyl content was calculated by the absorbance of the samples at 365 nm (Montazeri et al., 2023).

2.8. Malondialdehyde (MDA) assay

MDA is the main product of lipid peroxidation (LPO) in oxidative stress status which measured by thiobarbituric acid (TBA) and spectrophotometric method at 532 nm (Kakkar et al., 1995; Niki, 2008). For this test, 50 mg tissue sample was homogenate in 0.5 mL KCl (154 mM) and then 1.25 mL TCA (20 %) was added to prepared sample. Then supernatant was discarded following centrifuged at 3500 rpm for 10 min and 1.25 mL fresh sulfuric acid (0.05 M) and 1 mL of freshly prepared TBA reagent contained TCA(5 %) + HCl (0.5 N) + TBA (0.3 %) was added to sediment, mixed and transferred to boiling water (>96 °C) for 30 min. After that, the tubes were moved into ice and after cooling, 2 mL of n-butanol was added to each tube and mixed. Finally, samples were centrifuged at 3500 rpm for 10 min and the n-butanol layer was separated and the absorbance was read at 532 nm (Anoush et al., 2022b; Bijani et al., 2022). Tetramethoxypropane (TMP) was

applied as the standard to draw a calibration curve and measure MDA content in μmol/g tissue.

2.9. Histopathological assessment

The formation of Ab deposits in hippocampus and cortex plays a critical role in manifestation of AD-related spatial memory dysfunction (Edwards et al., 2014). The brain of different treated groups was fixed in 10 % formalin. The dehydration process was done using sequential dehydration using 50, 70, 80, 90 % and absolute alcohol. After the clearing and paraffin fixation of the tissue, 5–7 μm slices were prepared whole of the brain and installed on slides. Hematoxylin and eosin staining (H&E) was performed, and the pathological alterations were studied under optical microscope (Zeng et al., 2013).

2.10. Statistical analysis

Results were expressed as mean ± SD, and R studio programming software was used for statistical analyses. Six rats were used in behavioral tests and 3 rats in molecular tests. Comparisons between the groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of Empagliflozin on cognitive performance

As shown in Fig. 2, the random swimming patterns of all study design groups are shown in Morris water task. Our results in Fig. 3 showed that the therapeutic effects of empagliflozin (EMPA) and Donepezil (DON) were investigated on the test day. The presented data in Fig. 3 revealed that in animal treated with scopolamine induce memory impairment and significantly decline in Q_2 time percentage compared to normal animals ($F_{7,56}$=23.807; $P < 0.001$). Also, donepezil (DON) as the standard accepted treatment for AD and EMPA (4 mg/kg) demonstrated no significant difference compared to the control group. In addition, ALZ+ EMPA (10 mg/kg) animals showed a significant rise in the presence of the Q_2 zone compared to the AD group ($P < 0.01$).

Furthermore, the control, DON, EMPA (4 mg/kg), and the ALZ+ EMPA (4 mg/kg) groups did not differ significantly from each other in time spent on the main track of their swimming in the target zone (Q_2); while the pattern of swimming for rats that received scopolamine was observed to be randomized with a significant decrease in the time spent in Q_2 ($P < 0.001$; Fig. 3). Although, post-treatment with EMPA (10 mg/kg) demonstrated a significant increase in the Q_2 time percentage ($P < 0.001$). Therefore, it supposed that DON and EMPA (4 mg/kg) could effectively inhibit scopolamine's destructive and harmful effects on the cholinergic system by improving the function of spatial memory.

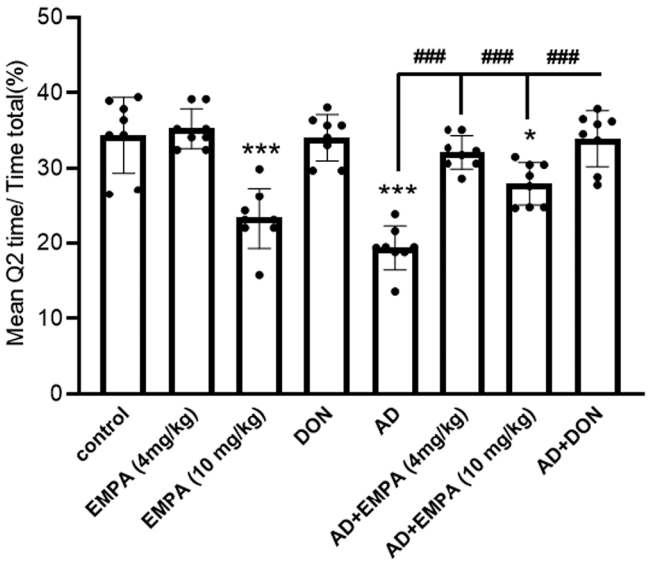

Fig. 3. The effect of Empagliflozin and Donepezil on Q_2 time/total time percentage. Values are expressed as the mean± SD and were analyzed using one-way ANOVA followed by Tukey's post hoc test (n = 8). * $p < 0.05$ as compared to control rats; * * $p < 0.01$ as compared to control rats; * ** $p < 0.001$ as compared to control rats; # $p < 0.05$ as compared to AD rats; ## $p < 0.01$ as compared to AD rats; and ### $p < 0.001$ as compared to AD rats.

3.2. Effect of Empagliflozin on GSH levels

The one-way ANOVA analysis indicated a remarkable decrease in the GSH level in AD rats ($P < 0.001$). On the other hand, compared to the control group, treatment with donepezil (DON) and EMPA (4 mg/kg) in

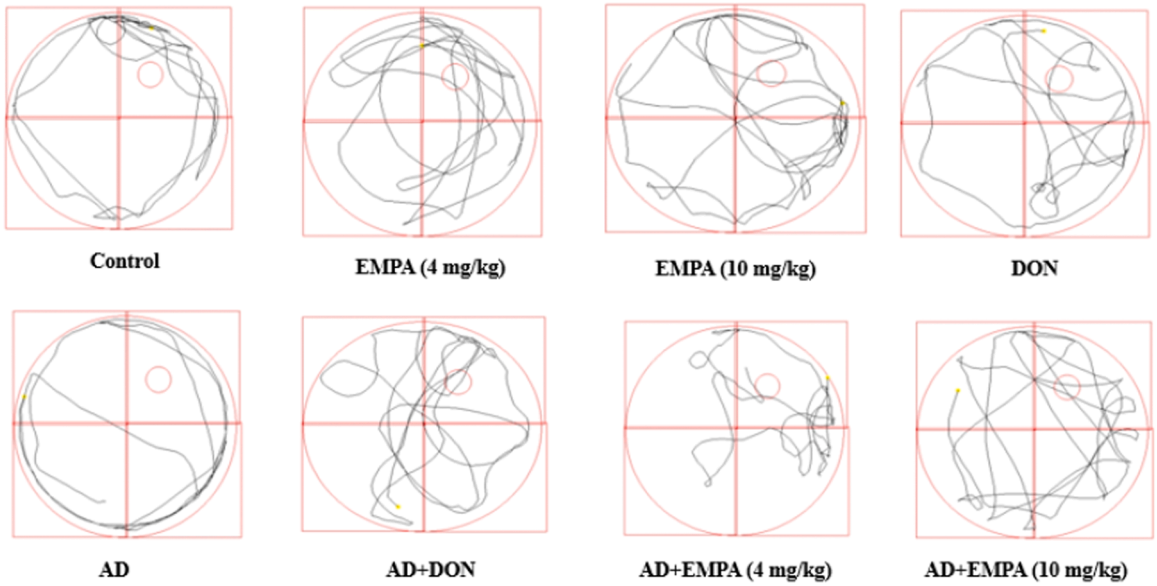

Fig. 2. Randomized swimming patterns of selected rats in the water maze task.

AD rats demonstrated no significant change in GSH levels. Results of EMPA (4 mg/kg) + AD rats demonstrated a notable increment in GSH level compared with AD groups, although the significant rise was observed in AD+DON rats ($P < 0.01$).

3.3. Effect of Empagliflozin on FRAP antioxidant marker

Analysis of FRAP data as an antioxidant capacity biomarker demonstrated significantly decline in total antioxidant power levels in AD rats compared with control group ($t = 36.216$, $P < 0.001$, Fig. 4b) without any significant difference in donepezil (DON) and EMPA (4 mg/kg) treated normal animals as compared with control mice. One-way ANOVA analysis revealed a significant increase in the FRAP level of in AD+ DON and AD+EMPA (4 mg/kg) group compared to AD treated rats ($P < 0.01$; Fig. 4b). Furthermore, it was found that the level of FRAP in AD+EMPA (10 mg/kg) was equal to that of AD rats.

3.4. Effect of Empagliflozin on protein carbonylation (PCO) and MDA levels

The results of MDA levels as the main product of lipid peroxidation revealed that a significant increase of MDA level was observed in AD rats in comparison with control groups ($t = 14.676$, $P < 0.001$, Fig. 4c). However, there was no significant difference between the MDA levels of control rats treated with donepezil (DON), EMPA (4 mg/kg) and EMPA (10 mg/kg) that of control group. Statistical Analysis revealed, that MDA level in AD animals treated with donepezil (DON), EMPA (4 mg/kg) and EMPA (10 mg/kg) was significantly lowered compared to AD animals ($P < 0.001$).

The post-hoc analysis declared significantly higher levels of protein carbonyl levels as a protein carbonylation biomarkers with AD treated rats compared to control group ($t = 4.544$, $P = 0.006$, Fig. 4d). Statistical analysis of PCO levels demonstrated that EMPA (4 mg/kg), EMPA (10 mg/kg) and DON (positive control) treated groups in AD animals is similar to control rats which can significantly decrease its amount in contrast with AD group ($P < 0.05$). Furthermore, EMPA (10 mg/kg) treated AD rats have created non-significant difference in PCO level as compared with the AD group.

3.5. Effect of Empagliflozin on histological appearance

As shown in Fig. 5, moderate to severe alteration in basophilic necrotic neuron, vacuolization and microglial nodule in ALZ treated rats compared to control groups. Also, we observed no difference between EMPA (4 mg/kg) and EMPA (10 mg/kg) with control animals, while the intense of pathologic alternation was decreased to normal to mild microglial nodule in ALZ+ EMPA (4 mg/kg), ALZ+EMPA (10 mg/kg), as well as ALZ+DON (1 mg/kg) treated rats compared to ALZ treated

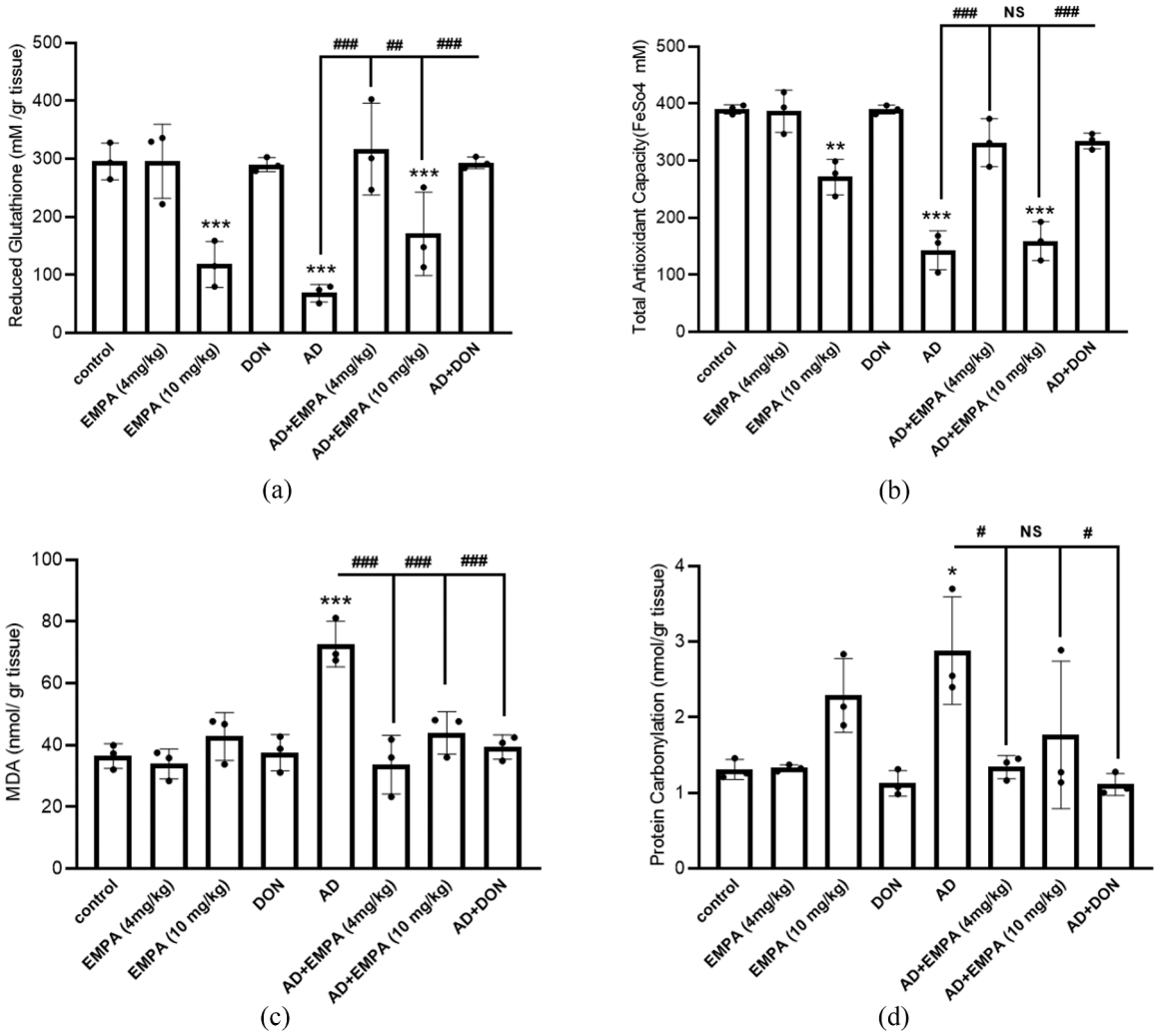

Fig. 4. The effect of Empagliflozin and Donepezil on (A) reduced glutathione (B) total antioxidant (C) MDA (D) PCO level. Values are expressed as the mean ± SD and were analyzed using one-way ANOVA followed by Tukey's post hoc test (n = 3). * $p < 0.05$ as compared to control rats; * * $p < 0.01$ as compared to control rats; * ** $p < 0.001$ as compared to control rats; # $p < 0.05$ as compared to AD rats; ## $p < 0.01$ as compared to AD rats; and ### $p < 0.001$ as compared to AD rats.

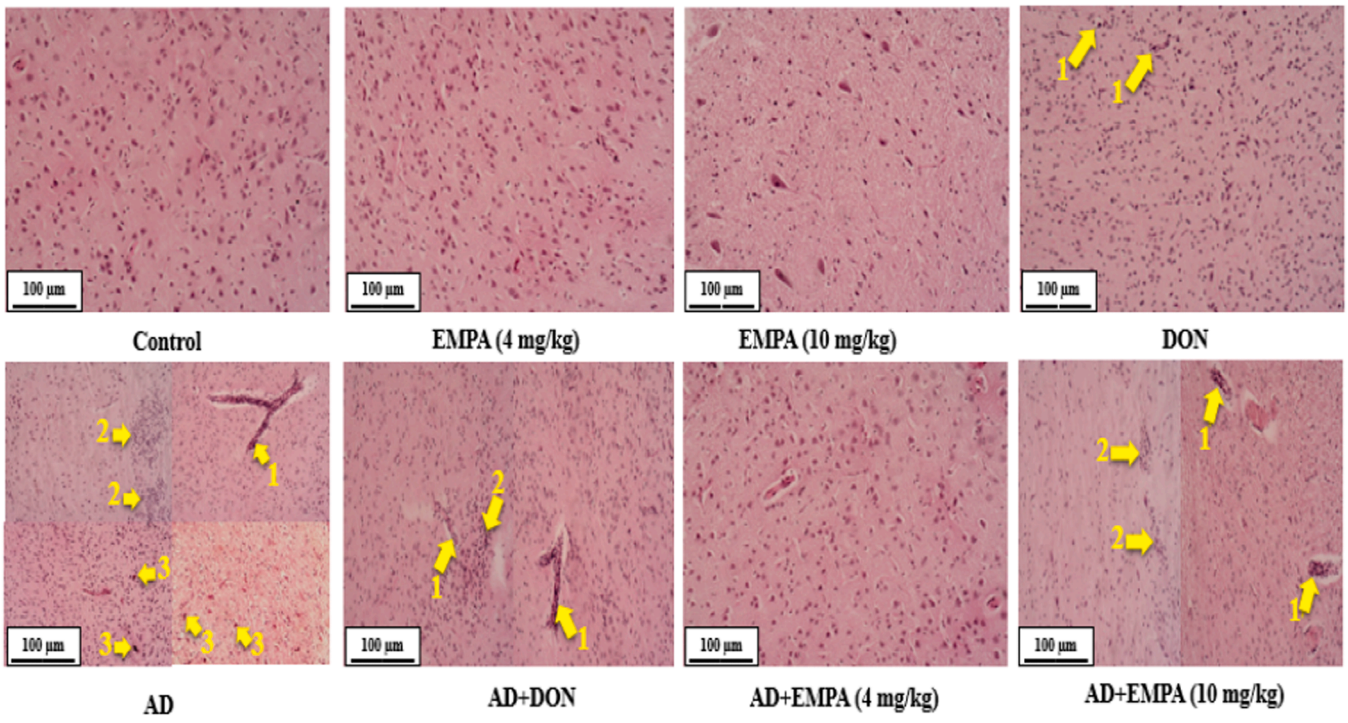

Fig. 5. H&E staining images of cerebral cortex tissue in experimental groups. (A).Control group (B). EMPA (4 mg/kg) (C). EMPA (10 mg/kg) without no damage (D) ALZ group: High level of Peri-Vascular inflammation (1), Accumulation of lymphoid cells (2) and Deposition of amyloid plaques (3) is equal to grade 3; (E). ALZ+ EMPA (4 mg/kg): no damage; (G). ALZ+ EMPA (10 mg/kg): Mild level of peri-Vascular inflammation and accumulation of lymphoid cells is equal to grade 1; (H). ALZ+DON: Moderate level of peri-Vascular inflammation and accumulation of lymphoid cells is equal to grade 1 or 2.

rats (Fig. 4). It seems that the positive therapeutic effect of EMPA (4 mg/kg) is better than EMPA (10 mg/kg) and DON in AD induced rats.

4. Discussion

In this study, Wistar rats underwent Scopolamine induced AD-like disorder, which has caused memory impairment, MDA surge and cellular antioxidant depletion, the treatment with 4 or 10 mg/kg of EMPA by gavage for 8 days has improved memory function, biochemical markers and histopathological characteristics with relative effect to Donepezil. The potency of 4 mg/kg of EMPA treatment was significantly higher than 10 mg/kg applied dose, since the memory function and antioxidant capacity improved more noticeably. this aspect is one of the remarkable aspects of this study.

T2DM is associated with increasing the risk of AD, as it increases the risk of brain damage and cognitive impairment. Though the effect of drugs causing hypoglycemic episodes have been proved in decreasing the chance of AD, the direct effect of such medications in controlling cognitive disorder and oxidative disorders in brain has yet to be proven (Barbagallo and Dominguez, 2014). Multiple natural extracts and antioxidants, including coenzyme Q10, melatonin, Vitamin E, Vitamin C and Selenium have demonstrated memory function recovery effect on rodents induced AD-like disorder (Ali et al., 2021; Juszczyk et al. 2021). T2DM medications namely, glibenclamide and metformin have demonstrated AD ameliorating effects, namely modulating total plasma tau, brain amyloid β 42 and indirect acetylcholinesterase inhibition effects (Ali and Ali, 2022). Sodium glucose co-transporter-2 (SGLT-2), as a family of medications inhibiting reabsorption of glucose in early proximal tubule. The neuroprotective effect of SGLT-2 agents has been studied on ischemic stroke, and Liraglutide and Empagliflozin demonstrated more potent neuro protective effect in comparison with metformin (Simanenkova et al., 2022).

In this study, 4 mg/kg of EMPA treatment has managed to improve memory performance in relative effect to 1 mg/kg DON group. In related study, obese mice which went under fat diet, and shown consequence memory impairment, were treated with EMPA which induced neuronal projection development and synaptic plasticity improvement via effecting dopaminergic pathways (Chen et al., 2023). EMPA treatment has also been capable in normalizing neural MDA and PCO production as markers of cellular ROS over production and mitochondrial malfunction, and recovering GSH level demonstrating probable indirect antioxidant effect via modulation of pro-inflammatory marker or improvement in mitochondrial electron chain transfer (Schönberger et al., 2023).The exact underling biomarkers that lead to the improvement in brain biochemical factors and memory performance by EMPA treatment still requires further investigation.

The endogenous antioxidants like GSH and CoQ_{10} play a key role in progressive behavior of neurodegenerative disorders, especially AD (Andalib et al., 2019; Raj Rai et al., 2021; Anoush et al., 2022b). In AD as an age dependent neurodegeneration disease, the soluble Aβ mitigates the cysteine/ GSH pathways which highly manifested itself in prefrontal and hippocampus performance (Paul, 2021). The EMPA has shown potential in replenishing neural GSH in AD-like disorder model utilized in this study.

The application of EMPA in former studies have managed to reduce infarct volume size and nuclear pyknosis in brain with effect stronger than Gliclazide (Amin et al., 2020). In this study, the appearance of dark neurons is an indication of neurodegeneration and neural death, commonly noticed under long-term cholinesterase inhibitors exposure, which has been prevented by EMPA treatment (Ahmadpour et al., 2019). The microglia aggregation is commonly noticed when neural degeneration is present, EMPA treatment managed to lower the count of these nodules frequency (Vinters and Kleinschmidt-DeMasters, 2018). Studies have indicated enlargement of pre-vascular space as an early marker of AD progression, they act as a part of glymphatic system act in clearance of waste material form neural tissue (Lynch et al., 2022). In this study, 4 mg/kg EMPA application has lowered the frequency of pre-vascular enlarged spaces. The deposition of amyloid plaques attracts

the immune cells into the neural site, in short-term phagocytes help increase the clearance of Aβ plagues, however in elderly, the over secretion of inflammatory cytokines and the higher rate of Aβ formation leads to higher neuro inflammation and neural apoptosis (Hohsfield and Humpel, 2015). In this study, both Aβ formation and lymphocyte accumulation has been reversed by 4 mg/kg EMPA treatment.

In conclusion, administration of 4 and 10 mg/kg EMPA on Scopolamine induced memory impairment rodent model has significantly improved memory function. The underling manifestation of cognitive performance amelioration were modulation of MDA and PCO as markers of oxidative stress and rebalancing GSH level as the main neural endogenous antioxidant.

Study limitations

For further investigations it is appropriate to measure both aspects of memory function, since the episodic memory also plays an important part in cognitive behavior of patients with AD, and this function can be investigated by mean of novel object recognition test. In case of spatial memory, hippocampus and cortex are both important in its functionality. The provision of H&E staining data for the hippocampus in future studies will offer additional correlation with the spatial behavior.

Since oxidative markers are the first markers of neurodegeneration, this study mainly focuses on free radicals and antioxidant content in brain. However, many systems are contributed in pathophysiology of AD including, immune response, inflammatory response and nitrate metabolites. The effect of EMPA treatment on these systems can be further studied to present a more in-depth view of the neuroprotective mechanism of this substance.

The anti-diabetic agents can alter the consumption of food/water by the rodent, and alternation in weight of rodents could be utilized as an influencing factor, so it is recommended these measurements to be carried out in future studies.

The findings of this study indicated while higher dose EMPA (10 mg/kg) application showed less potency in recovering memory performance and antioxidant depletion, administration of EMPA (10 mg/kg) on non-AD induced rats has significantly dropped the aforementioned factors compared to control. This affect may be due to low treatment window of EMPA in neural function, which we highly recommend the future investigation to use more narrower treatment dose choices.

Ethics approval

All procedures were carried out following the regulations of the University and the Guide for the Care and Use of Laboratory Animals of National Institutes of Health (Ethical Code: ZUMS.REC.1399.177) and Guide for the Care and Use of Laboratory Animals (eighth edition, National Academies Press). Full efforts were made to reduce animals' use and advance their welfare.

Authors' contributions

MJH and MA contributed in the study design, supervised the research, analyzed the data and edited the manuscript. SB, NT, ZHB and FR performed the experiments and data collection and prepared manuscript drafting. Histopathological assessment was done by AKH. MJH, MA and SB revised the language and grammar of the manuscript. All authors read and approved the final manuscript.

Disclosure statement

The authors declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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CRediT authorship contribution statement

Ali kalantari-Hesari: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Formal analysis, Data curation. **Mir-Jamal Hosseini:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology, Investigation. **Mahdieh Anoush:** Writing – original draft, Visualization, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Neda Taghaddosi** and **Fatemeh Rahmati:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Data curation. **Zahra Hosseini-Bokaei:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Soroush Bijani:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

The data that support the findings of this study are available on request from the corresponding author, [Mir-Jamal Hosseini]. The data are not publicly available due to our critical plan to investigate different studies based on our design.

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