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Sodium-glucose co-transporter inhibitor dapagliflozin attenuates cognitive deficits in sporadic Alzheimer's rat model

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ABSTRACT: Alzheimer's disease (AD) and Type 2 Diabetes Mellitus (T2DM) are both characterized by similar pathologies, and studies have shown that various drugs from both groups may be effective in another. The effects of sodium-glucose co-transporter (SGLT)2 inhibitors in AD are unknown. According to molecular docking studies, various SGLT inhibitors have acetylcholinesterase (AChE) inhibition activity, which is therapeutic target for AD. In this study, we investigated the effects of SGLT2 inhibitor dapagliflozin on intracerebroventricular (icv) streptozotocin (STZ) induced sporadic AD rats using open field test (OFT), novel object recognition test (NORT), passive avoidance test (PAT) and Morris's water maze test (MWMT).

Rats were randomly divided into 4 groups: vehicle-control, icv STZ, dapagliflozin, and galantamine treatments groups. STZ was injected bilaterally in two divided doses on day 1 and 3. All treatments began on day 1 and continued to day 21. OFT was performed for evaluating animal locomotor activity and anxiety. Other behavioral tests, NORT, PAT, and MWMT was performed for determining of learning and memory ability of rats. On day 21, all rats were decapitated.

Our results showed that treatments with dapagliflozin and galantamine significantly prevented learning and memory deficits in behavioral tests. Dapagliflozin may present as a potent dual inhibitor of SGLT2 and AChE. Our results may form the basis of future dual treatment against diabetes and diabetes-related neurological diseases. The effects of possible dual AChE and SGLT2 inhibition by a single compound may help to establish new drugs that perform both anti-AD and antidiabetic action.

KEYWORDS: Dapagliflozin; Alzheimer's disease; type 2 diabetes mellitus; sodium-glucose co-transporter inhibitor.

1. INTRODUCTION

Alzheimer's disease (AD) is identified with increasing memory loss and other cognitive functions and it is the most observed type of dementia. AD can be categorized as late-onset sporadic AD (SAD) and early-onset familial AD (FAD). Most cases of AD are multifactorial SAD which includes several etiopathogenic mechanisms. In addition to many risk factors affecting AD; neuroinflammation, head trauma, impaired brain glucose/energy metabolism, diabetes mellitus (DM) and the presence of Apolipoprotein E (ApoE)-ε4 allele are among the risk factors for AD [1].

AD is mainly characterized by the formation of particles in senile plaques and neurofibrillary tangles (NFTs) [2]. The senile plaques consist mainly of amyloid beta (Aβ) and the NFTs consist of hyperphosphorylated tau (p-tau) proteins. It has been shown that cell loss is associated with increased impaired oxidative stress and mitochondrial dysfunction, oxidative stress, and cerebrovascular disease or cerebral hypoperfusion. In addition, it was shown that the damaged process of glucose uptake in brain and energy metabolism defects accompany the early stages of AD [3].

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There is a rising interest in clarifying the role of insulin resistance, hyperinsulinemia, and type 2 diabetes mellitus (T2DM) in the pathogenesis of AD, cognitive impairment, and neuronal cytoskeletal lesions associated with AD and Aβ accumulation in the brain [4-5]. Increasing evidence suggests that T2DM is an independent risk factor for SAD [6-7]. In many previous studies, intracerebroventricular (icv) streptozotocin (STZ) has been used to establish an AD-type neurodegeneration model in adult mice and rats [8-11]. STZ, a glucosamine-nitrosurea derivative, causes DM by specifically destroying beta cells in the islets of the pancreas when applied to the periphery. In addition to the lack of understanding of the cytotoxicity mechanism of STZ, it is known that the alkylating properties of STZ metabolites produce reactive oxygen species, causing oxidative stress and DNA damage [12].

While STZ damages pancreatic beta cells as well as another glucose transporter (GLUT)2 expressing organs, single or double icv STZ injection(s) chronically reduces cerebral glucose uptake and produce many other effects, including molecular, pathological, and behavioral properties of AD [13]. These effects led to the use of icv STZ to form a neurodegeneration model. In adult rats, icv STZ causes a chronic decrease in glucose and glycogen metabolism (10-30%) in the brain [8]. These effects are associated with significantly reduced brain energy balance and oxidative metabolism [9], inhibition of insulin receptor function 11], and progressive deficiencies in learning, memory, cognitive behavior [11-15]. Therefore, this model provides the biochemical and physiological abnormalities that occur in AD. It has been shown that icv STZ may mimic brain insulin resistance has negative effects on cognitive function in patients with DM and AD [16].

Cholinergic system hypofunction may trigger dementia or worse in AD because it plays an important role in learning and memory processes of the central cholinergic system [17]. Acetylcholine (ACh) is essential for the functioning of cholinergic delivery to regulate and enhance learning and memory processes. Many studies with mice and rats have shown that the icv STZ model increases acetylcholinesterase (AChE) activity [18-19].

On the other hand, AD and T2DM are very similar pathologies, and several studies have shown that various drugs from both groups may be effective in another [20-27]. The effects of sodium-glucose co-transporter (SGLT)2 inhibitors, which have recently received Food and Drug Administration (FDA) approval for T2DM are unknown on AD.

Studies with dapagliflozin [28], empagliflozin [29], and canagliflozin [30], SGLT2 inhibitors, have shown that these drugs improve cognitive function. In the study investigating the cognitive efficacy of dapagliflozin, vildagliptin, and their combination; all treatments in obese rats reduced insulin sensitivity, mitochondrial ROS production, mitochondrial membrane potential change, as well as inflammation and apoptosis. Only dapagliflozin of treatments improved the deterioration of hippocampal synaptic plasticity. The improvement in the learning-memory shown in Morris' water maze test (MWMT) has been associated with these findings [28].

Another aspect that makes SGLT inhibitors remarkable for AD was found in molecular docking studies. According to molecular docking studies, various SGLT inhibitors perform AChE inhibition as well as SGLT inhibition [31-33]. In a molecular docking study with dapagliflozin, it has been shown that the hydrophobic and cation-π interactions of dapagliflozin play an important role in the correct positioning of dapagliflozin in the catalytic site of the SGLT2 and AChE enzyme. Thus, it was reported that dapagliflozin may act as a potent dual inhibitor of SGLT2 and AChE. It is stated that the described results may form the base of future dual treatment against DM and DM-related neurological disorders [31].

The effects of SGLT2 inhibitor dapagliflozin on depression and anxiety were investigated by our team for the first time. According to this research, antidepressant [34] and anxiolytic [35] effects of dapagliflozin were found. The antidepressant effect of dapagliflozin was evaluated with the forced swim test and it was found that when dapagliflozin was administered acutely, it showed an antidepressant-like effect [34]. Anxiolytic effect was evaluated by open field test (OFT). According to this test, when dapagliflozin was administered acutely, it increased the time spent in the center and decreased the number of grooming as the anxiolytic effect [35].

As an antidiabetic SGLT2 inhibitor of dapagliflozin, it is thought to have effects on AD with its acetylcholinesterase activity due to its structural properties along with regulation glucose metabolism. In this study, we investigated the effects of dapagliflozin on AD in the icv STZ induced SAD model using novel object recognition test (NORT), passive avoidance test (PAT), and MWMT by comparing it with the galantamine which is the FDA-approved AD drug.

2. RESULTS

2.1. Effects of Dapagliflozin on Open Field Test

We used OFT to measure the locomotor activity of rats (Figure 1). There is no significant difference between groups on squares crossed in OFT. In the present study, no significant difference was found between different groups on locomotor activity. This result eliminates the probability that the locomotor activity of the rats may itself contribute to changes in cognitive tests, particularly the tasks in the MWMT.

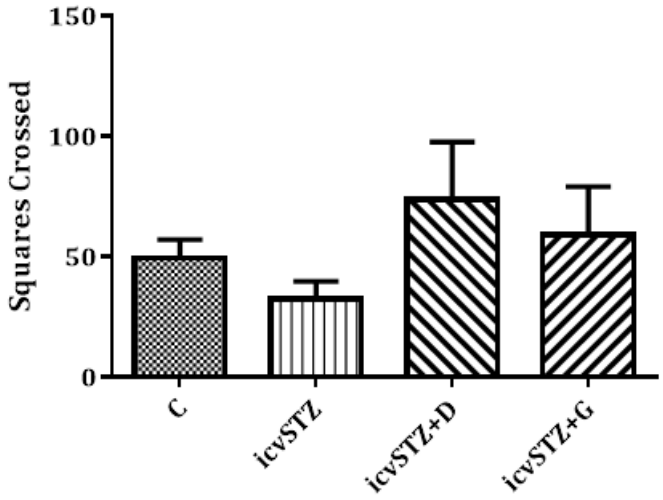

Figure 1. Squares crossed of rats in open field test. Values are expressed in Mean±SEM (n=8). One-way ANOVA with Tukey post-test was performed using GraphPad Prism version 6.05 for Windows. (C: Vehicle-control group, icvSTZ: intracerebroventricular Streptozotocin group, icvSTZ+D: Dapagliflozin treatment group, icvSTZ+G: Galantamine treatment group.)

2.2. Effects of Dapagliflozin on Novel Object Recognition Test

When control group was examined in the novel object recognition test, there was a significant difference ($p<0.05$) between the exploration time of familiar and novel object; when STZ group was examined, no significant difference was found between the exploration time of familiar and novel objects (Figure 2). Besides, the discrimination index ($p<0.05$) and the preference index ($p<0.05$) were shown to be decreased in the STZ group compared to the control group (Figure 3). These results demonstrated a short-term memory impairment in icv STZ rats.

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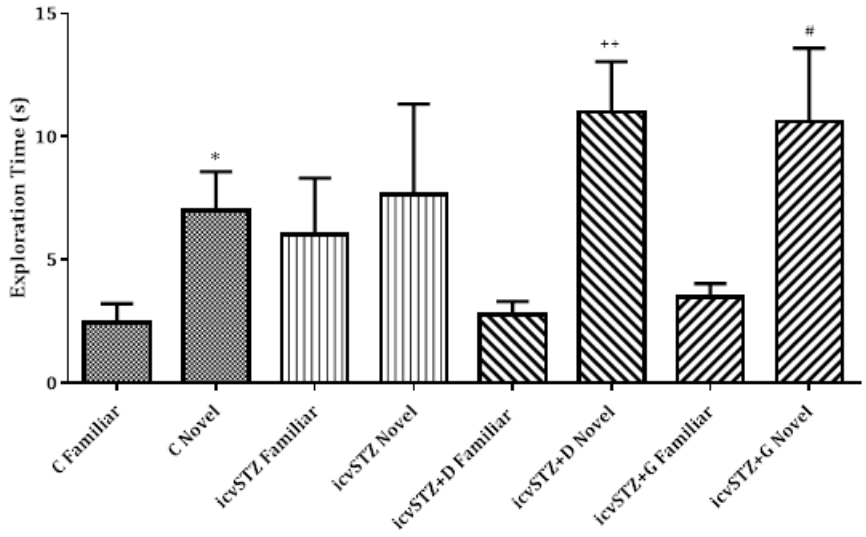

Figure 2. Exploration time of rats in novel object recognition test. Values are expressed in Mean±SEM (n=8). One-way ANOVA with Tukey post-test using GraphPad Prism version 6.05 for Windows. *p<0.05 in comparison with C Familiar, ++p<0.01 in comparison with icvSTZ+D Familiar, #p<0.05 in comparison with icvSTZ+G Familiar. (C: Vehicle-control group, icvSTZ: intracerebroventricular Streptozotocin group, icvSTZ+D: Dapagliflozin treatment group, icvSTZ+G: Galantamine treatment group.)

In the treatment groups, it was seen that there is a significant difference between the exploration time of familiar and novel objects (Figure 2). Also, STZ+Dapagliflozin group (p<0.01) was found to be significantly higher than STZ+Galantamine group (p<0.05). The discrimination index increased on STZ+Dapagliflozin group compared to STZ group (p<0.05) (Figure 3a). The preferential index increased on STZ+Dapagliflozin group (p<0.01) and STZ+Galantamine group (p<0.05) compared to STZ group (Figure 3b). These results suggest that short-term memory impairment improved in the treatment groups compared to STZ group.

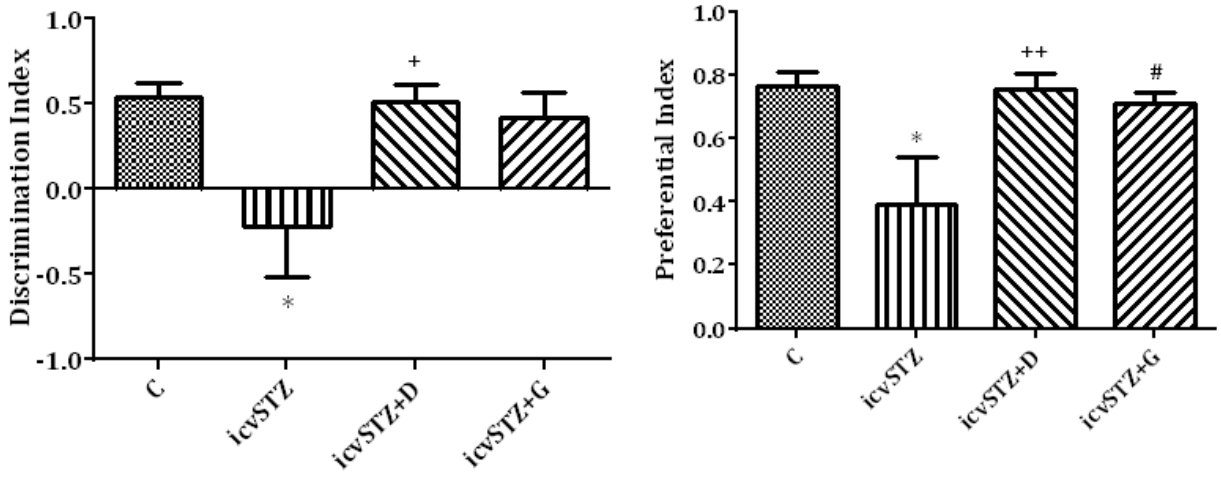

Figure 3. Discrimination index (a) and preferential index (b) of rats in novel object recognition test. Values are expressed in Mean±SEM (n=8). One-way ANOVA with Tukey post-test was performed using GraphPad Prism version 6.05 for Windows. a: *p<0.05 in comparison with C and +p<0.05 in comparison with icvSTZ. b: *p<0.05 in comparison with C, ++p<0.01 in comparison with icvSTZ and #p<0.05 in comparison with icvSTZ. (C: Vehicle-control group, icvSTZ: intracerebroventricular Streptozotocin group, icvSTZ+D: Dapagliflozin treatment group, icvSTZ+G: Galantamine treatment group.)

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3.3. Effects of Dapagliflozin on Passive Avoidance Test

In the PAT, the step-through latency time in STZ group decreased ($p<0.05$) when compared to control group (Figure 4). The reduced capacity in remembering to encounter electric shock following entrance into the dark compartment. When treatment groups were examined, no significant change was observed compared to STZ group.

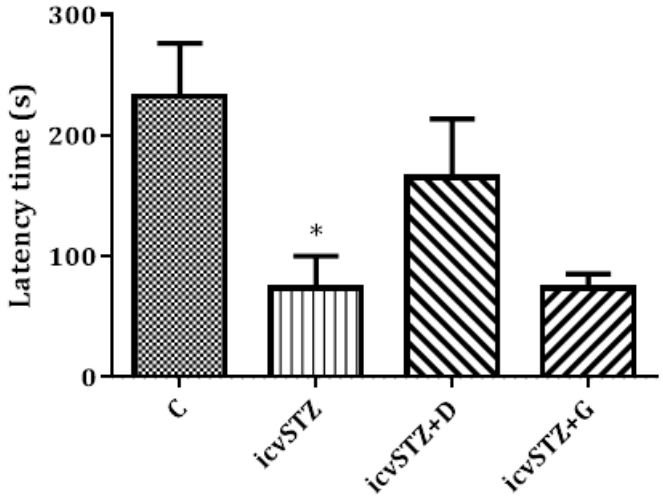

Figure 4. Step-through latency of rats in passive avoidance test. Values are expressed in Mean±SEM (n=8). One-way ANOVA with Tukey post-test was performed using GraphPad Prism version 6.05 for Windows. *p<0.05 in comparison with C. (C: Vehicle-control group, icvSTZ: intracerebroventricular Streptozotocin group, icvSTZ+D: Dapagliflozin treatment group, icvSTZ+G: Galantamine treatment group.)

2.4. Effects of Dapagliflozin on Morris' Water Maze Test

During the training phase, there was a decrease in latency to find the platform underwater in all rats, this result shows that all rats learned the platform. However, it was observed that icvSTZ group needs more time for training. These results indicate that there is a deterioration in the coding and remembering of the spatial memory (Figure 5).

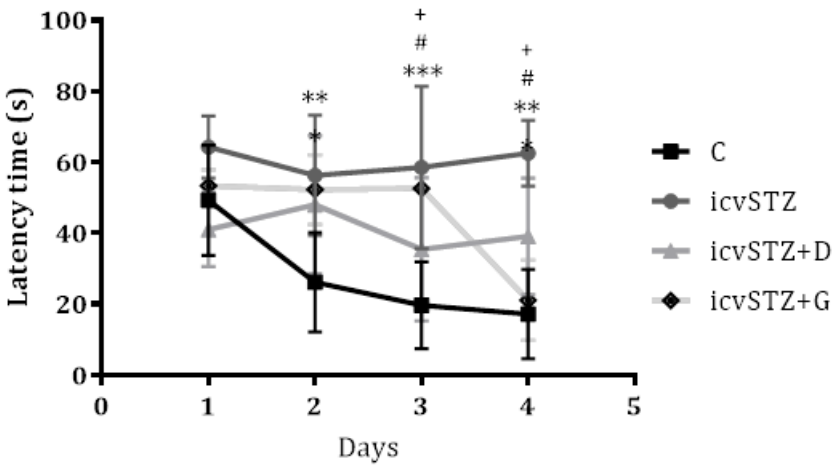

Figure 5. Latency to platform of rats in Morris' water maze test. Values are expressed in Mean±SEM (n=8). One-way ANOVA with Tukey post-test was performed using GraphPad Prism version 6.05 for Windows.

**p<0.01 in comparison with C (icvSTZ), ***p<0.001 in comparison with C (icvSTZ), #p<0.05 in comparison with icvSTZ (icvSTZ+D) and +p<0.05 in comparison with icvSTZ group (STZ+G). (C: Vehicle-control group, icvSTZ: intracerebroventricular Streptozotocin group, icvSTZ+D: Dapagliflozin treatment group, icvSTZ+G: Galantamine treatment group.)

3. DISCUSSION

SAD is a late-onset disease and usually develops after the age of 65 years [14]. The insulin-resistant brain condition with anomalies in glucose/energy metabolism and abnormal signaling in insulin plays a central role in neurodegeneration in SAD [3]. The icv STZ rat model has been proposed to produce cognitive deficits similar to those seen in SAD [36]. Therefore, in our study, the icv STZ rat model was used to mimic SAD. The phenotypes of icv STZ rats associated with DM and brain insulin resistance were shown to be at high risk for the development of AD-like pathology and cognitive decline [37].

In most studies, the effects of icv STZ on brain function and behavior were observed in relatively short period of time, such as several weeks [13]. In one of the most recent reviews [38], the cognitive and neurochemical changes triggered by icv STZ injection follow a three-stage time-dependent model: acute response develops within the first 1 month; there is a tendency to return to normal values within 1 to 3 months; and finally, in 6 to 9 months, the decompensation phase takes place slowly and gradually. On the basis of all these, we preferred the model with two times icv STZ administration on day 1 and 3 of the study which will last for 21 days.

NORT is a cognitive test that estimates non-spatial visual episodic memory and is based on the behavior of rodents in discovering objects [39]. In our study, as shown in previous studies, it has been shown that icv STZ administration disrupts learning and memory in exploratory activity, discrimination index and preferential index in NORT. Our treatment groups, dapagliflozin and galantamine, enhanced this deterioration.

Rats, naturally prefer dark places to bright places, and this preference is changed with a single conditioning session in the PAT [40]. Many previous studies have found that the step-through latency time associated with learning-memory is reduced in the PAT [41-42]. In our study, icv STZ was also found to reduce latency time. However, no significant difference was found in the treatment groups compared to icv STZ group.

MWMT, one of the common tests to assess the cognitive function of rodents in behavioral studies, was used in this study as a model for assessing spatial learning and memory [43]. The inability of the rats to learn the platform in the labyrinth in this test shows that they cannot remember and encode spatial information which is a characteristic of cognitive failure due to brain disruption [3]. Some brain regions and neurotransmitter systems such as the hippocampus, striatum, basal forebrain, brain cortex, and cerebellum have been shown to play an important role in the MWMT performance of rodents [39, 43]. Moreover, avoiding water in the maze serves as a motivation for the test and is advantageous as it eliminates the use of other motivational stimuli such as food and water deprivation. Additionally, water provides a homogeneous environment and defeats interference from odorous cues [43]. In the present study, a significant decrease in the learning time during the 4 training days in the control group indicated that normal memory results were obtained, while the increase in the time spent on the target quadrant to investigate the platform removed during the probe test indicates that the memory is normal [14]. Different studies have shown that icv STZ causes cognitive impairment in the rodent MWMT [44-48]. In our study, icv STZ caused the time to reach the platform to be higher than other groups and increased the time to reach the target quadrant and decreased the time spent on the target quadrant. These findings show that icv STZ causes memory impairment. When the treatment groups are examined, it is seen that the time to reach the platform is shorter on training days, the time it takes to reach the target quadrant on the probe test is shortened and the total time spent on the target quadrant increases. In our study, we demonstrated that the devastating effect of icv STZ on acquisition learning was reversed by dapagliflozin. These results indicate that treatment with dapagliflozin may prevent memory loss in the brain of rats exposed to STZ-induced cognitive failure.

AChE inhibition is the main target for AD treatment [49]. AChE is also known to play a role in human neocortical neuroplasticity processes [50]. There is some critical structural and functional confirmation associating AChE to AD: a part near the C-terminus in the AChE structure is poorly similar to the N-terminus of the Aβ peptide [51]. In a comparative study on neurotoxicity, AChE complexed human Aβ peptide fibrils

showed greater toxicity than uncomplexed Aβ peptide fibrils. AChE can increase both amyloid deposition and toxicity of this deposition [52].

Most AD studies have focused on the expression of choline acetyltransferase (ChAT), a synthase enzyme for ACh, and AChE because ACh levels and energy metabolism are reduced in early AD. Rats treated with icv STZ also show decreased levels of AChE. Increased levels of AChE expression in the icv STZ brain may cause an increase in ACh, thereby exacerbating ACh deficiencies caused by a decrease in ChAT expression. Since AChE is expressed in many cell types, including glia, the significantly increased astrocyte population in icv STZ brains may result in increased expression of AChE in this model. It is also interesting to find reduced ChAT expression in the icv STZ model because insulin stimulates ChAT, ChAT regulates Ach biosynthesis, and icv STZ disrupts insulin signals in the central nervous system [12].

Many studies have shown that icv STZ causes cognitive deficits in rodents identified using behavioral and biochemical analyses. In these tests, it was observed that icv STZ administration increased AChE level activity. Furthermore, in rats icv-STZ reduces the synthesis of acetyl-coenzyme A (acetyl-CoA) and consequently causes the cholinergic system has an important role in the learning and memory process, so hypofunction of cholinergic system may trigger memory loss and confusion in Alzheimer's disease, or may be worse [53]. It has been reported that cholinomimetic drugs such as donepezil improve memory deficiency and anticholinergic drugs such as scopolamine cause amnesia in animals [54-55]. ACh is essential for the proper functioning of cholinergic delivery to balance and develop learning and memory processes. The synthesis of ACh depends on the presence of acetyl-CoA and insulin, which regulate the activity of ChAT [18].

Inhibition of SGLT2 serves as a novel approach to diminish hyperglycemia independent of insulin secretion or effect and also demonstrates a new pharmacotherapy for the treatment of T2DM [56-57]. SGLT2 is one of the best targets in the treatment of DM. AChE has long been considering a therapeutic target for AD. Thus, dapagliflozin may present as a potent dual inhibitor of SGLT2 and AChE. The results expressed may form the basis for future dual treatment against DM-related neurological diseases. The effects of possible dual AChE and SGLT2 inhibition by a single compound may help to establish new drugs that display both anti-AD and antidiabetic action. Dapagliflozin can represent as a dual inhibitor for AChE and SGLT2. Importantly, since the development of DM is associated with AD, the design of new AChE inhibitors based on antidiabetic drug scaffolds would be particularly useful. It is estimated that dapagliflozin may be an effective dual inhibitor of AChE and SGLT2. Both hydrophobic and cation-π interactions play an equally important role in the correct positioning of dapagliflozin with AChE and SGLT2 to allow the basis of a future dual treatment against DM-related neurological disease [31].

In a study [28], the effect of dapagliflozin on peripheral insulin sensitivity, brain insulin sensitivity, hippocampal synaptic plasticity, brain mitochondrial function, brain inflammation, brain apoptosis and cognitive function in the obese-insulin resistant state were investigated. According to the results of this study, the possible mechanisms underlying dapagliflozin in the recovery of cognitive function in high fat diet-induced obese rats are to restore peripheral insulin sensitivity, to substantially reduce brain oxidative stress, to improve brain mitochondrial function, and to restore brain inflammation; in addition to insulin signaling has been shown to reduce brain apoptosis leading to increased brain insulin sensitivity and hippocampal synaptic plasticity.

In an animal study with another SGLT2 inhibitor, canagliflozin, it was shown that canagliflozin improves memory-impaired function by affecting cholinergic and monoaminergic systems in the scopolamine-induced amnesia model [30]. In this study, it was also found that canagliflozin reduces cerebral AChE activity in obese diabetic rats.

In a study performed with empagliflozin, it was found that empagliflozin reduced cerebral oxidative stress DNA oxidative damage in db/db mice. In the same study, it has been shown that empagliflozin increases cerebral brain-derived neurotrophic factor (BDNF) level and it is thought that empagliflozin increases BDNF and improves oxidative stress, which is responsible for improving cognitive damage [29].

The reversal of STZ dementia with dapagliflozin may be due to the AChE inhibitory and neuroprotective effect of dapagliflozin, which maintains ACh levels in the synaptic cleft. Based on our behavioral results, it can be said that the proposed interaction with molecular modeling for the dapagliflozin compound is in full agreement with the experimental affinity and is similar to a classical anticholinesterase such as clinically used galantamine.

4. CONCLUSION

According to our results, treatments with dapagliflozin and galantamine significantly prevented learning and memory deficits in behavioral tests. Dapagliflozin may present as a potent dual inhibitor of SGLT2 and AChE. Thus, the improvement in cognition is probably due to the effects of dapagliflozin on SGLT2 inhibition, as well as inhibition of AChE and its effect on neuronal plasticity and oxidative stress. It is important to clarify the mechanism of these effects in future studies.

5. MATERIALS AND METHODS

5.1. Chemicals

STZ was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Dapagliflozin and galantamine were purchased from Sigma-Aldrich, Inc. (St. Louis, Missouri, USA)

5.2. Animals

All animal experiments were carried out with the approval of Marmara University Animal Experiments Local Ethics Committee (permission number: 38.2018.mar). Thirty-two adult female and male Wistar albino rats (250-350 g) (n=8 in each group) were obtained from Marmara University The Experimental Animal Implementation and Research Center. The rats were housed under controlled temperature (20±2 °C), in humidity (40-60 %) and light (12 h/12 h light/dark regime)-regulated rooms. The animals were kept on a standard rodent pellet diet, with tap water available ad libitum. All rats were kept in their cages for a one-week study to adapt conditions before starting the experiment.

5.3. Experimental Design of Study

The experimental design of study is shown in Figure 6. Rats were randomly divided into 4 groups:

- Vehicle-control group (C; healthy rats were given bilaterally icv citrate buffer in 5 µl volume on day 1 and 3 of study and intragastric distilled water on each day),
- icv STZ group (icvSTZ; Alzheimer rats were given bilaterally icv 3 mg/kg STZ with citrate buffer in 5 µl volume on day 1 and 3 of study and intragastric distilled water on each day),
- Dapagliflozin treatment group (icvSTZ+D; Alzheimer rats treated with dapagliflozin were given bilaterally icv 3 mg/kg STZ with citrate buffer in 5 µl volume on day 1 and 3 of study and intragastric 1 mg/kg Dapagliflozin [28] with distilled water on each day) and
- Galantamine treatment group (icvSTZ+G, positive control group; Alzheimer rats treated with 10 mg/kg galantamine were given bilaterally icv 3 mg/kg STZ with citrate buffer in 5 µl volume on day 1 and 3 of study and intragastric 10 mg/kg galantamine [27] with distilled water on each day).

All treatments began on day 1 of the experiment and continued to day 21. On day 21, the last day of study, all rats were decapitated.

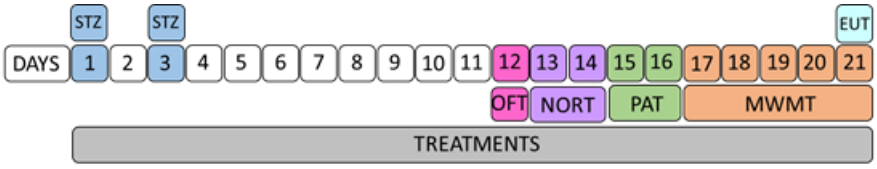

Figure 6. The experimental design of present study. STZ: streptozotocin, OFT: open field test, NORT: novel object recognition test, PAT: passive avoidance test, MWMT: Morris' water maze test, EUT: euthanasia.

5.4. Induction of Alzheimer: Intracerebroventricular Injection of STZ

Rats were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.; Pfizer) and xylazine hydrochloride (10 mg/kg, i.p.; Bayer). The scalp was shaved, cleaned and cut to reveal the skull. The rat was placed in the stereotaxic frame and a midline sagittal incision was made in the scalp. On both sides of the skull, holes were drilled using the following coordinates: 0.8 mm posterior to bregma, 1.5 mm lateral to sagittal suture and 3.6 mm beneath on brain surface. STZ (1.5 mg/kg of each hole, totally 3 mg/kg of each day) was injected bilaterally in two divided doses on days 1 and 3. The concentration of STZ in citrate buffer was adjusted to give a total of 3 µl of the solution to both holes. C animals received icv injection of the same volume

of citrate buffer on days 1 and 3. After the second injection, the skin was sutured and then daily antiseptic powder was applied [58-59].

5.5. Behavioral Tests

5.5.1. Open Field Test

OFT is one of the most accepted procedures for evaluating animal locomotor activity [60]. On day 13 of the study, the animals were put to OFT. OFT apparatus which rats have previously unknown consisted of square based Plexiglas box (50 x 50 bases, 40 cm high) and open from above. The ground is divided into 25 equal squares, each 10 x 10 cm and consisted of two regions: the peripheral region (10 cm from each wall in the area) and the central region. Each animal was placed and evaluated separately on the apparatus. The animals' behavior was recorded on video for 5 minutes and evaluated the number of squares passed by two researchers as double-blind. The number of squares passed was considered a measure of locomotor activity. The apparatus surfaces were cleaned with consecutive trials to eliminate any bias that might have occurred due to the odor of the previous rat [41].

5.5.2. Novel Object Recognition Test

We used the NORT to examine short-term memory. NORT is a relatively rapid behavioral test that can be used as a pre-screen. NORT's simple design utilizes the spontaneous behavior of rats to approach and discover novel objects with a naturally activating stimulus. When spontaneous behavior is examined, artificial stimulus, food deprivation, reinforcement and/or prior training are not required. NORT was performed in a 40 cm high walled black plexiglass open area (50 x 50 cm) at dimly lit condition on day 14 (habituation) and 15 (test). Rats were habituated to the NORT area without any object to acclimate to the environment for 30 minutes 24 hours before testing. In this test, object recognition was performed between the training phase and the test phase at 1-hour intervals. During the first trial, two identical objects were located in opposite corners and these objects are now called familiar (F). In the second trial, one of the objects was replaced by a novel (N) one. The sizes of identical and novel objects were comparable to each other. After each rat, the NORT apparatus should be cleaned with 70 % ethanol to remove any urine or scent cues. Exploration of identical and novel objects was defined if rats licked, sniffed or touched the object. Based on the exploration time of each object, the discrimination index [(N-F)/(N+F)] and preferential index [N/(N+F)] was calculated. Exploration time was scored by treatments blind researcher [39, 61].

5.5.3. Passive Avoidance Test

On days 16 and 17 were applied to acquisition and test of PAT, respectively. PAT is a fear-motivating test to examine long and short-term memories in a relational manner. In the test, the animal must behave in opposition to the innate darkness of the animal with the fear of being taught. The passage time of rats to the dark compartment was measured. In the test, a cut-off time was determined as 300 seconds if the animal did not cross to the dark compartment. The duration of the experiment is 300 seconds; if the rats do not pass into the dark compartment within 300 seconds, the experiment is terminated and the transition time is recorded as 300 seconds. The test apparatus consists of two compartments isolated with a retractable lid. One of the compartments is illuminated by a bright light, the other compartment has covered by dark opaque walls. The ground in both compartments is made of metal shocking grids. On the dark compartment, the ground is wired for 3 seconds to receive an electric shock of 0.5 mA.

On acquisition day, the rat was left in the bright compartment when the retractable lid between the compartments was closed. After rat passed into the dark compartment, the lid was closed and 0.5 mA electric shock was supplied. After the shock was given, the rat returned to the cage for the test session. On test day, the test trial was performed just like the previous day, but no foot shock was applied. In the test, a cut-off time was determined as 300 seconds if the animal did not cross to the dark compartment. After each rat, both compartments should be cleaned with 70 % ethanol [41].

5.5.4. Morris' Water Maze Test

We used MWMT to investigate spatial learning. MWMT consist of two parts; training was performed on day 18-21. MWMT apparatus consisted of a round stainless-steel tank. The tank is divided into 4 quarters with 4 fixed points around it and there are different shapes and colors 4 cues attached to the opposite of each direction. The tank contained the escape platform of the same color as the rest of the maze (to eliminate any false positives due to vision) in the middle of one of the quarters of the area during the training. In the trials, rats were released to water their faces rotate the wall from one of the directions and allowed 75 s for finding

the platform. If the rat did not find the escape platform within 75 s, it was lightly directed to the platform and allow to remain on it for 20 s. After 1 minute, rats were released to water another direction. Therefore, all rats were trained 16 times in 4 days, 4 different directions each day. The direction order was changed every day. Rats were subjected to learning, which was evaluated for reaching the platform [39, 41].

5.6. Statistical Analysis

The results of the tests were analyzed by one-way ANOVA and two-way ANOVA followed by the Tukey method as a post-test and represented as mean ± standard error of the mean (S.E.M). P values <0.05 were considered significant. Data analysis was performed using GraphPad Prism 6.5 software (San Diego, USA).

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