Genistein improves spatial learning and memory in male rats with elevated glucose level during memory consolidation

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Abstract

Cognitive dysfunction due to higher blood glucose level has been reported previously. Genistein (GEN) is a phytoestrogen that we hypothesized might lead to improved memory, despite elevated blood glucose levels at the time of memory consolidation. To investigate this hypothesis, we compared the effects of orally administered GEN on the central nervous system in normal versus glucose-loaded adult male rats. A battery of behavioral assessments was carried out. In the MAZE test, which measured spatial learning and memory, the time of normal rats was shortened by GEN treatment compared to the vehicle group, but only in the early stages of testing. In the glucose-loaded group, GEN treatment improved performance as mazes were advanced. In the open-field test, GEN treatment delayed habituation to the new environment in normal rats, and increased the exploratory behaviors of glucose-loaded rats. There were no significant differences observed for emotionality or fear-motivated learning and memory. Together, these results indicate that GEN treatment improved spatial learning and memory only in the early stages of testing in the normal state, but improved spatial learning and memory when glucose levels increased during memory consolidation.

Keywords: Genistein, Phytoestrogen, Glucose load, Oral administration, Rat, Spatial learning

1. Introduction

Genistein (GEN) is a naturally occurring phytoestrogen present in soy, with a higher binding affinity to estrogen receptors (ER) compared with other phytoestrogens [1–3]. It has been reported that GEN can be detected in the brain soon after intraperitoneal (i.p.) administration [4], suggesting that GEN can pass through the blood-brain barrier and affect the central nervous system (CNS).

A number of studies have shown that GEN has a neuroprotective or memory-improving effect in animal models of Alzheimer's disease [5] and global cerebral ischemia [6] and in ovariectomised (OVX) rats [7–9]. However, few studies have discussed the effects of GEN on learning and memory in the normal state. To understand the effects of GEN on neuronal functioning, it is important to study its effects in normal animals.

Diabetes is the most common serious metabolic disorder in humans[10], and is associated with long-term complications that affect the eyes, kidneys, heart, blood vessels and nerves [10]. Cognitive dysfunction due to diabetes have been reported previously [11,12]. A recent study revealed that the increased oxidative stress in diabetes produces oxidative damage in many regions of rat brain including the hippocampus [13].

It has been previously reported that GEN decreases plasma glucose levels in streptozotocin (STZ)-induced diabetic rats [14]. Furthermore, GEN also ameliorates hyperglycemia in a mouse model of type 2 diabetes [15]. This suggests that GEN might be an effective antidiabetic agent [16].

However, it has not been reported the effects of GEN on learning under the state that the blood glucose level is elevated. We thought that the investigation about this point will be important on thinking about the effects of GEN on prevention of the cognitive decline by the elevation of blood glucose level. Therefore, we performed simultaneous glucose load and GEN treatment in adult male rats. Specifically, this study employed a battery of behavioral tests to investigate the effects of GEN on the CNS in normal versus elevated blood glucose states, at the time of memory consolidation in male rats.

2. Materials and Methods

2.1. Animals

We used male Sprague-Dawley (SD) rats, which were obtained at 5 weeks of age from Kyudo Co. Ltd. (Kumamoto, Japan). All animals were maintained in a 12:12-h light-dark cycle (lights on from 0700 to 1900) at 22 ±2 ºC and 55 ±10% humidity.

The animals were food restricted (12 g/day food and 33.3 mL/day water per rat) from 6 weeks of age, to increase the motivation for reward in MAZE test which was an appetite-motivated maze test. Once a week, food restrictions were lifted to avoid an excessive reduction in body weight. Experimentations were conducted as follows (Fig. 1).

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University, with the approval of the Institutional Animal Care and Use Committee.

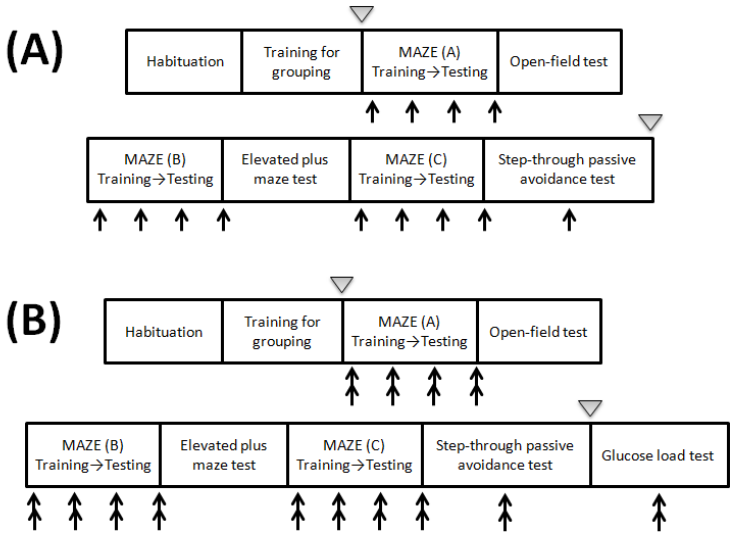

Fig. 1. Experimental procedures. Rats were received either oral administration only (A) or oral administration + intraperitoneal (i.p.) injection of 20% glucose solution (B) under the schedule of normal (A) or glucose-loaded group (B). The arrow and the inverted-triangle indicate the points of administration and measuring the fasting blood glucose level, respectively.

2.2. Drug administration

GEN was purchased from LKT Laboratories Inc. (Minnesota, USA). Rats were divided into six groups: three groups each of normal and glucose-loaded rats, comprising a vehicle, a 1 mg/kg/day of GEN (1 mg/kg GEN), and a 10 mg/kg/day of GEN (10 mg/kg GEN) group. Vehicle groups received 0.5% Carboxymethyl Cellulose Sodium Salt (CMC-Na; Wako Pure Chemical Industries, LTD., Osaka, Japan), while other groups were administered GEN dissolved in this solution. For the glucose-loaded groups, rats were administered 20% glucose solution to elevate blood glucose level (D (+)-glucose; Wako Pure Chemical Industries, LTD., Osaka, Japan) at a rate of 1 g/kg body weight. Glucose was administered by intraperitoneal injection to avoid gastric physical stimulation because the MAZE test, used subsequently, was an appetitive-motivated task. Oral administrations (1 mL/kg/day) of vehicle or GEN were conducted by feeding needles. After this administration, the glucose solution was administered immediately. All administrations were performed under light anesthesia using halothane (Fluothane, Takeda Pharmaceutical Co. Ltd., Tokyo, Japan).

For the MAZE test, drugs were administered within 30 min following training or each testing. For the open-field and elevated plus maze test, drugs were administered the day before the tests. In the step-through passive avoidance test, drugs were administered within 30 min following the training session.

2.3. The open-field test

We assessed locomotor activity, emotionality, and exploratory behavior in rats using the open-field test, following the method previously described by Hall [17]. The open-field apparatus had a circular bottom 60 cm in diameter, and an enclosing wall 50 cm high. The floor of the apparatus was illuminated by a light (100 W) placed 80 cm above the floor, and was divided by black lines into 19 equal regions (Fig. 2). The form of divisions was slight different, but its area was mostly same. The open-field consisted of an inner circle (placed 12–30 cm from the wall) and an outer ring (placed 0–12 cm from the wall). Rats were placed on the center of the floor, and then ambulation (total number of times the black lines were crossed), inner-cross (number of times the black lines were crossed in the inner circle), and rearing (number of times the rat stood up on its hind legs) were counted for 3 min. These events were measured three times, with a two-hour interval. Ambulation, inner-cross, and rearing were used as indices of locomotor activity, emotionality, and exploratory behavior, respectively. The test was

conducted when rats were 8 weeks old.

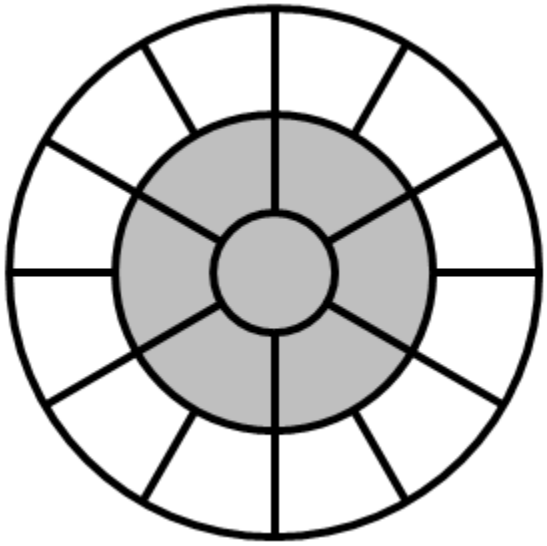

Fig. 2. Bottom view of the open-field apparatus. Gray area shows the inner circle.

2.4. The elevated plus maze test

Anxiety was measured using the elevated plus maze test [18]. This maze consisted of apparatus in the form of a plus sign, with two closed arms surrounded by walls 60 cm in height and two open arms (no wall), placed 60 cm above the floor. Each arm was 50 cm × 10 cm, painted black, and connected to a central neutral zone (14 cm × 14 cm). In each trial, rats were placed in the neutral zone facing an open arm, and the total number and total time spent in each arm were measured for a period of 5 min. The elevated plus maze test was conducted at 10 weeks of age.

2.5. The MAZE test

The MAZE test was used to assess spatial learning and memory, as described previously [19]. This apparatus consisted of a large compartment (90 × 90 × 50 cm) with an attached goal partition (15 × 15 × 50 cm). The inside of the apparatus was painted white, and was illuminated by three bulbs (100 W) from 100 cm above the floor. To

facilitate memory of the route to the goal, four different markers were attached to the wall. We used three types of apparatus —MAZE (A), MAZE (B), and MAZE (C)—the distance until the goal become longer as mazes were advanced (Fig. 3). Each maze consisted of divider plates of various sizes (50 cm × 15 cm, 50 cm × 30 cm, 50 cm × 45 cm, and 50 cm × 60 cm) combined within the large compartment. The test was appetite-motivated, with 300 μL of milk as the reward, placed in the goal partition. MAZE (A) was performed at 8 weeks, MAZE (B) at 10 weeks, and MAZE (C) at 12 weeks of age.

The procedure of the MAZE test followed the order: Habituation → Training for grouping → Training → Testing. "Habituation" was carried out in order to habituate seven-week-old rats to the maze apparatus and reward, and was performed for three consecutive days. Following habituation, "Training for grouping" was conducted, which resulted in rats being divided into administration groups based on their movement ability. For every MAZE test, "Training" was then performed in order for rats to learn the correct route to the goal compartment. The apparatus used in this step blocked off incorrect routes within the maze, and the time from the start to attaining the reward was measured, within 3 min. "Testing" was carried out a day after "Training." The spatial learning ability of rats was tested three times per day, for three consecutive days. In testing, time and error were recorded for a maximum period of 5 min. Time was defined as the latency required to get the reward. Error was defined as the number of entries to an incorrect area. Both parameters were recorded until rats get the reward or become a time limit.

For both the training and testing sessions, the rat was placed on the start point at the beginning of the trial, and allowed to find the milk reward. If the rat could not reach the goal within the time limit, it was guided to it. After each trial, the rat was removed from the maze and placed in a cage to rest for 1 min, before the next trial began. Experimenters wiped the floor of the MAZE apparatus after each trial in order to remove any odors.

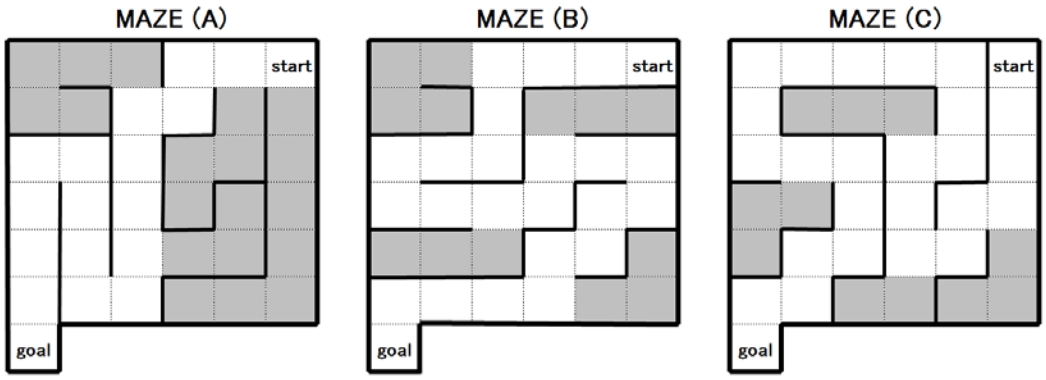

Fig. 3. Apparatus of the MAZE test. Three types of MAZE were employed. In these figures, white areas represent correct ways to the reward, while gray areas represent the error areas.

2.6. The step-through passive avoidance test

Fear-motivated learning and memory were assessed using the step-through passive avoidance test [20]. This apparatus (Shintecno Co. Ltd., Fukuoka, Japan) consisted of an illuminated chamber (10 × 20 × 12 cm) and a dark chamber (30 × 30 × 30 cm), connected by a path (8 × 8 cm). A guillotine door separated the two chambers, and an electric current was passed through a grid floor in the dark chamber.

The step-through test was conducted over three consecutive days in 13-week-old rats. On day 1, a rat was placed in the light chamber for 10 s for the habituation step. The pathway between chambers was then opened, and the rat was able to move freely between the chambers for 90 s. On day 2, a rat was again placed in the light chamber, and if it entered the dark chamber, it received a 5 s electric shock (1 mA) to the feet, using a shock generator (MSG-001, Toyo Sangyo Co. Ltd., Toyama, Japan). For this step, the latency to enter the dark chamber for the first time was recorded for each rat (the acquisition time). On day 3, the latency (retention time) was also measured, for a maximum period of 5 min.

2.7. Fasting blood glucose level

Fasting blood glucose levels were measured using One Touch® Ultra Vue™ (Johnson & Johnson K.K., Tokyo, Japan) in the both states in order to estimate the change in fasting blood glucose level due to GEN. Measurements were conducted prior to drug administration and after completion of all behavioral tests. Blood was collected from a

tail vein using a disposable needle. Because the period of food restriction after completion of behavioral testing was longer than the period before treatment, the fasting blood glucose level after behavioral testing might have decreased. To account for this, a decrease ratio of fasting blood glucose level was calculated as [decrease ratio (%) = (blood glucose level on the day before treatment − blood glucose level on the day all behavioral tests were completed) / blood glucose level on the day before treatment ×100].

2.8. Glucose load test

A glucose load test was performed at 14 weeks of age, and blood glucose level was measured at 30, 60, and 120 min after administration in the same way as described above. At the time of the glucose injection, oral administration of vehicle or GEN was also performed. Fasting blood glucose level was also measured prior to the glucose load test.

2.9. Statistical analysis

All results were analyzed using the two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests (Stat View, SAS, Cary, NC, USA). Results of behavioral tests for the normal and glucose-loaded groups were analyzed separately, and were considered statistically significant at $P < 0.05$. All data are presented as mean ± SEM.

3. Results

3.1. The open-field test

For the normal rats, ambulation reduced from 0 h to 2 h in all treatment groups (Fig. 4A). Whereas ambulation in the vehicle group decreased greatly in 2 h, ambulation in the GEN treated groups decreased slightly over time (Fig. 4A). A significant difference ($P < 0.05$) was observed at 2 h between the 1 mg/kg GEN group and the vehicle group (Fig. 4A). For inner-cross, the GEN treated groups showed slightly increased values at all measured points compared to the vehicle group, but these differences were not significant (Fig. 4B). For rearing, there were differences between the GEN treated groups and the vehicle group, but these differences were not significant (Fig. 4C).

In the glucose-loaded groups, the 1 mg/kg GEN group showed higher values and 10 mg/kg GEN group showed lower values for ambulation compared to the vehicle group; however, these differences were not significant (Fig. 4D). The low-dose GEN group moved more in the inner circle than the vehicle group, but this difference in behavior was also not significant (Fig. 4E). Rearing frequency in the GEN treated groups showed higher values than the vehicle group at all measured points, and these differences were significant at the 0 h time point (1 mg/kg GEN group: $P < 0.01$, 10 mg/kg GEN group: $P < 0.05$) (Fig. 4F).

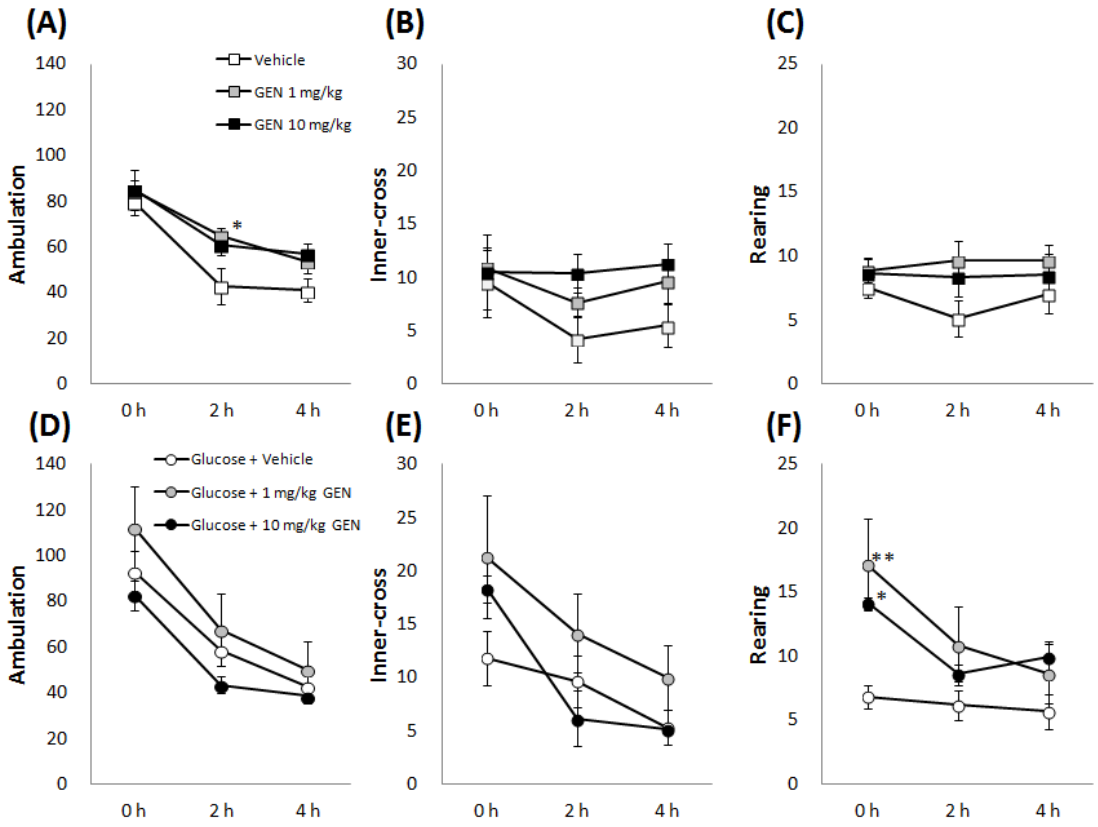

Fig. 4. The open-field test. Rats received either oral administration (A, B, C: normal, n = 8 per group), or oral administration + intraperitoneal (i.p.) injection of 20% glucose solution (D, E, F: glucose-loaded, n = 6 per group). Ambulation (A, D) was measured as the total number of crossings, inner-cross (B, E) was measured as the number of crossings inside the inner circle, and rearing (C, F) was measured as the frequency of upright stances on the hind-legs. Results are expressed as mean ± SEM. *P < 0.05 and **P < 0.01 indicates a significant difference compared to the vehicle group.

3.2. The elevated plus maze test

In the normal rats, the number of entries into both arms was slightly decreased following GEN treatment compared with the vehicle group, but this was not significant (Fig. 5A). All groups showed similar time spent in both arms, and changes due to GEN were not observed (Fig. 5B).

In the glucose-loaded group, open arm entries for the 1 mg/kg GEN group, and closed arm entries for the GEN treated groups increased compared to the vehicle group, but

these differences were not significant (Fig. 5C). For the 1 mg/kg GEN compared with the vehicle group, time spent in the open arms was slightly longer, and in the closed arms was slightly shorter, but there were no significant differences between the two groups (Fig. 5D). For the 10 mg/kg GEN group, the time spent in both arms was equivalent to the vehicle group (Fig. 5D)

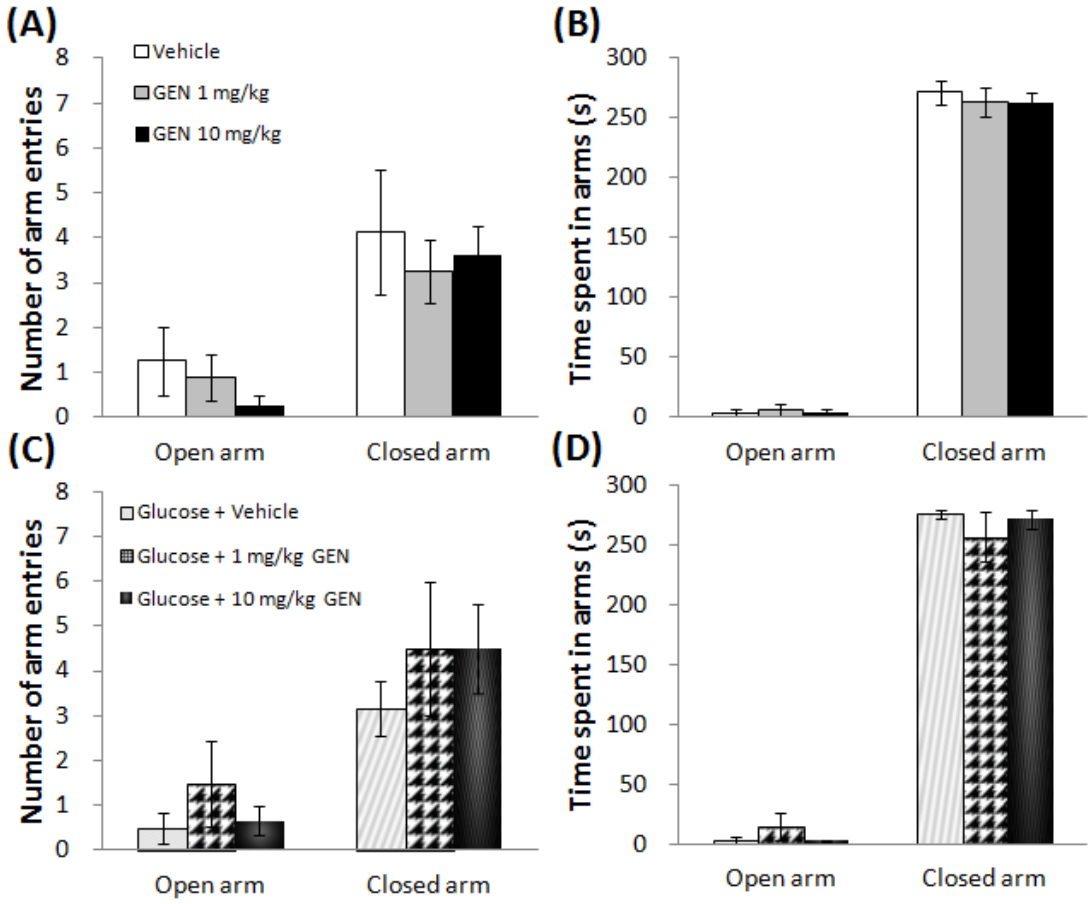

Fig. 5. The elevated plus maze test. Rats received either oral administration only (A, B: normal, n = 8 per group), or oral administration+ intraperitoneal (i.p.) injection of 20% glucose solution (C, D: glucose-loaded, n = 6 per group). The figure shows the number of arm entries (A, C) and time spent (B, D) in each arm. The results are expressed as mean ± SEM.

3.3. The MAZE test

In the normal rats, the rats which received 1 mg/kg GEN demonstrated decreased time in all mazes compared to the vehicle group, although a significant difference ($P<$ 0.05) was only observed for day 1 of MAZE (A) (Fig. 6A). In the 10 mg/kg GEN group, although the time was significantly shorter compared to the vehicle group in MAZE (A) (day 1 and day 3: $P<$ 0.01; day 2: $P<$ 0.05), the time increased gradually over time, and was similar to the vehicle group for MAZE (C) (Fig. 6A). On day 3 of MAZE (A), a significant difference ($P<$ 0.05) was also observed between the 1 mg/kg and 10 mg/kg GEN groups (Fig. 6A). Errors for both GEN treated groups were very similar to the vehicle group on MAZE (A) and (B) (Fig. 6B). For MAZE (C), the GEN treated groups showed slightly higher values than the vehicle group, but no significant differences were observed between any groups for errors (Fig. 6B).

In the glucose-loaded groups, the time for rats in the 1 mg/kg GEN group was almost equivalent to the vehicle group in MAZE (A), however the group showed decreased latencies compared to the vehicle group in MAZE (B) and (C), and significant differences were observed in MAZE (C) (day 2: $P<$ 0.01; day 3: $P<$ 0.05) (Fig. 6C). The time for the 10 mg/kg GEN group were also significantly shorter than the vehicle group in MAZE (B) ($P<$ 0.05) and (C) ($P<$ 0.01) (Fig. 6C). On day 3 of MAZE (A), the time taken by the 10 mg/kg GEN group was longer than the other groups, and this difference was significant ($P<$ 0.05) when compared to the 1 mg/kg GEN group (Fig. 6C). For the number of errors, the 1 mg/kg GEN group made fewer errors in MAZE (A) and (C), and the 10 mg/kg GEN group made fewer errors than the vehicle group in all MAZE tests (Fig. 6D). A significant difference ($P<$ 0.05) between the vehicle group and 10 mg/kg GEN group was observed on day 3 of MAZE (C) (Fig. 6D).

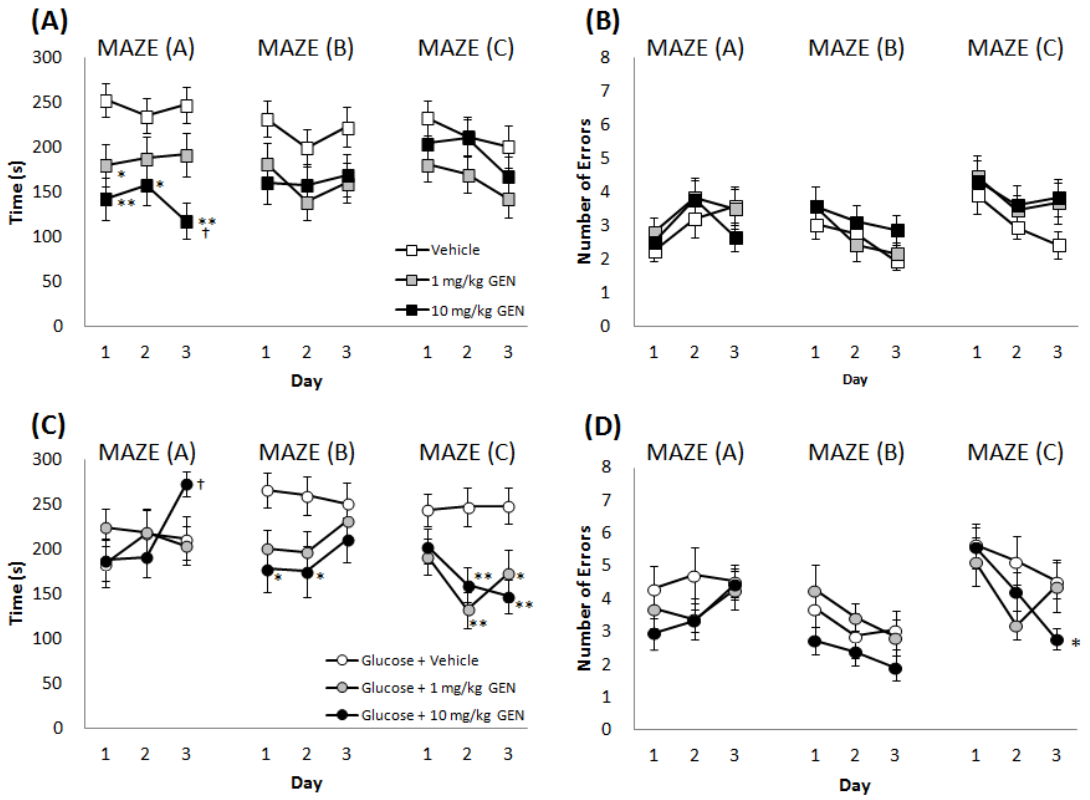

Fig. 6. The MAZE test. Rats received either oral administration only (A, B: normal, n = 8 per group) or oral administration + intraperitoneal (i.p.) injection of 20% glucose solution (C, D: glucose-loaded, for vehicle and 10 mg/kg GEN groups n = 6 per group, for 1 mg/kg GEN group n = 6 for MAZE (A) and (B), for MAZE (C), n = 5). The figure shows the time to reach the goal of the milk reward (A, C), and the number of incorrect entries (B, D). Time and errors are averaged over three trials for each testing day. The results are expressed as mean ± SEM. *$P < 0.05$ and **$P < 0.01$ indicate significant differences compared to the vehicle group, and † $P < 0.05$ indicates a significant difference compared to the 1 mg/kg GEN group.

3.4. The step-through passive avoidance test

There was no significant difference between the normal groups in the training session (Fig. 7A). In the retention trial, the 1 mg/kg GEN group showed slightly shorter latency than the vehicle group, but this difference was not significant (Fig. 7A). The latency for the 10 mg/kg GEN group was almost equivalent to the vehicle group (Fig. 7A).

For the glucose-loaded groups, there was no significant difference between groups

during training (Fig. 7B). In the retention trial, latencies were shorter for the GEN treatment groups compared to the vehicle group, especially for the 1 mg/kg GEN group, but no significant differences were observed (Fig. 7B).

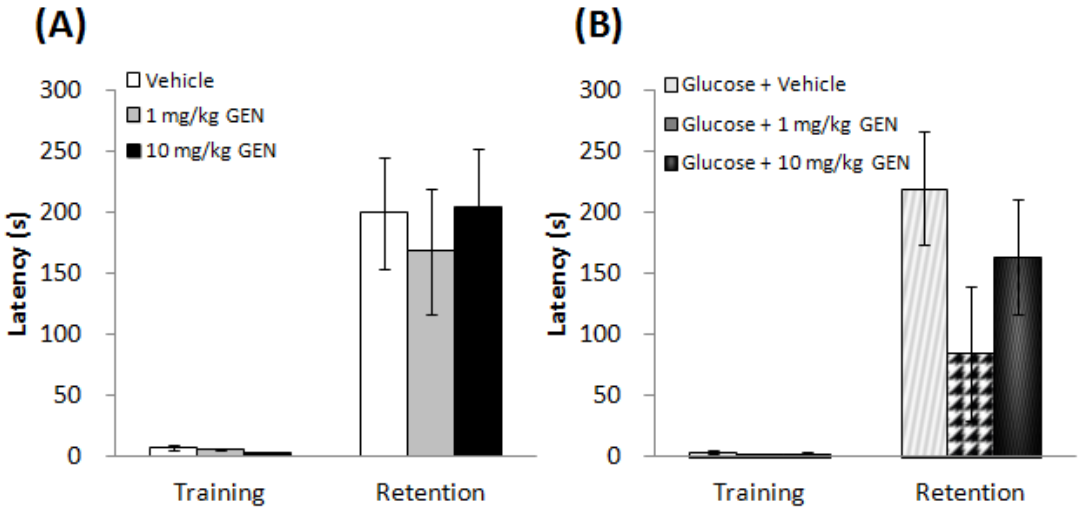

Fig. 7. The step-through passive avoidance test. Rats received either oral administration only (A: normal, n = 8 per group) or oral administration + intraperitoneal (i.p.) injection of 20% glucose solution (B: glucose-loaded, n = 6 for vehicle and 10 mg/kg GEN groups, n = 5 for 1 mg/kg GEN group). Figure shows latency to enter the dark chamber for the training and retention trials. Results are expressed as mean ± SEM.

3.5. Fasting blood glucose level

There were no significant differences in fasting blood glucose levels between groups at either of the time points. In the normal rats, the decrease ratios from the day before administration to the day behavioral tests were completed were 25.5 ±3.20, 24.8 ±3.99, and 21.1 ±3.87, for the vehicle group, 1 mg/kg GEN group, and 10 mg/kg GEN group, respectively. In the glucose-loaded groups, the decrease ratios were 12.1 ±5.16, 19.0 ±7.37, and 19.7 ±3.13, for the vehicle group, 1 mg/kg GEN group, and 10 mg/kg GEN group, respectively.

3.6. Blood glucose level after the glucose load test

The glucose load test was conducted in rats assigned to the glucose-loaded group only. Blood glucose level in the vehicle group was significantly increased ($P < 0.01$) at 30 min, compared to the group value at 0 min (Fig. 8), and a significant increase ($P < 0.05$) was also observed in the 10 mg/kg GEN group at 30 min compared to 0 min (Fig. 8). However, a significant increase in blood glucose level at 30 min after the glucose load was not observed in the 1 mg/kg GEN group (Fig. 8). There were no significant differences in blood glucose levels for any groups at any other time points when compared to 0 min (Fig. 8).

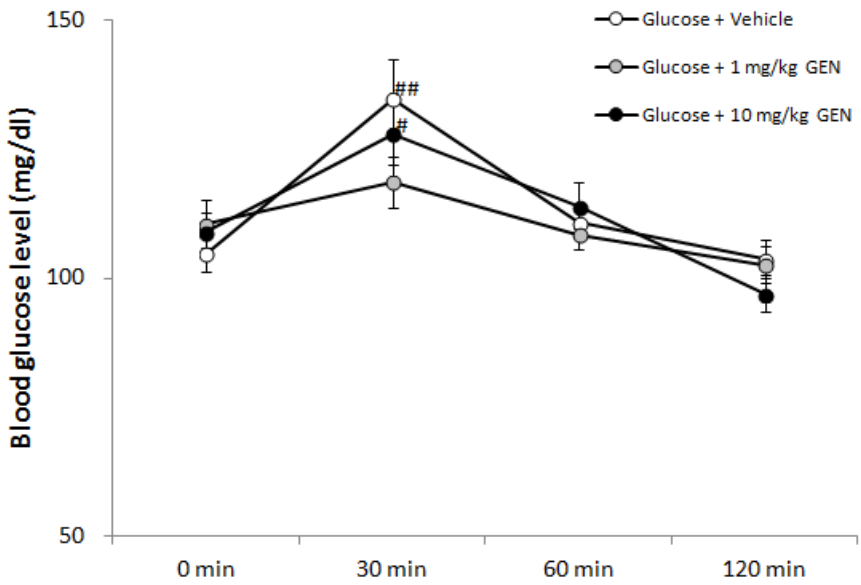

Fig. 8. Blood glucose levels for the glucose-loaded group only. Rats were orally administered drugs and injected 20% glucose solution (i.p.) after the first measurement of fasting blood glucose level and subsequent levels were measured 30, 60, and 120 min after the glucose load. Results are expressed as mean ± SEM. Vehicle and 10 mg/kg GEN groups (n = 6 for each group) and 1 mg/kg GEN group (n = 5). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ indicate significant differences compared to the glucose level measured at 0 min for each group.

4. Discussion

In the present study, we investigated the acute treatment effects of GEN on the CNS, in a normal state versus an elevated blood glucose state, on memory consolidation in adult male rats.

This study used dosage values of 1 mg/kg/day and 10 mg/kg/day. The low dosage of GEN (1 mg/kg/day) is an attainable daily amount, especially for Japanese people [21,22]. High doses of GEN (10 mg/kg/day) have been reported as having an improving or neuroprotective effect on brain function [5,23,24]. We have also previously indicated that these doses improved spatial learning and memory in rat offspring, using perinatal GEN treatment [25].

In the open-field test, the GEN treated groups in the normal state showed a higher level of ambulation than the vehicle group at 2 h and 4 h (Fig. 4A). In particular, the 1 mg/kg GEN group showed a significant difference ($P < 0.05$) at 2 h compared to the vehicle group (Fig. 4A). These data suggest that rats receiving GEN treatment take additional time to habituate to a new environment. However, in the glucose-loaded groups, there were no significant differences between groups in either ambulation or inner-cross (Fig. 4D, E), GEN treatment led to a significant increase in amount of rearing (1 mg/kg GEN group: $P < 0.01$; 10 mg/kg GEN group: $P < 0.05$) (Fig. 4F), indicating that GEN treatment in an elevated blood glucose state leads to greater curiosity in a new environment.

In the elevated plus maze test, GEN treated groups in normal rats did not show any significant differences for the number of arm entries or time spent in the arms (Fig. 5A, B). In the glucose-loaded groups, open arm entries, and time spent in open arms increased in the 1 mg/kg GEN group when compared to the vehicle group, although these differences were not significant (Fig. 5C, D). These results indicate that GEN treatments do not affect anxiety in either a normal or an elevated blood glucose state.

The MAZE test, which was designed in our laboratory, is a good method for observing changes in spatial learning and memory ability over time. In the normal rats, the time of the vehicle group was slightly decreased over time (Fig. 6A). This observation indicated that the vehicle group performed the good spatial learning curve with time. On the other hand, in the glucose-loaded vehicle group, the time increased from MAZE (A) to MAZE (B), and these longer time remained for MAZE (C) (Fig. 6C). A recent study has suggested that higher plasma glucose levels, even within the normal range, are associated with hippocampal atrophy in humans [26]. Another report has also suggested that higher fasting blood glucose levels are associated with reduced memory ability and

hippocampal microstructure in older women [27]. These data indicate that the hippocampus, which is involved in spatial learning, is sensitive to higher blood glucose levels. It has been reported that the cognitive system involving short- and long-term spatial learning and memory is impaired even in prediabetic rats, when accompanied by alterations to hippocampal glutamatergic neurotransmission and abnormal glucocorticoid signaling [28]. In contrast, it has been reported that glucose administration (250 mg/kg, i.p.) administered 30 min before the start of testing enhances cognitive performance in male SD rats [29]. In that study, cognitive function was measured at one time point using spontaneous alternation tests. In the context of the present study, the glucose injection might provide nourishment for the brain in the early stages of administration, but the extension of an elevated blood glucose level at the time of memory consolidation might bring about a decline in spatial learning and memory performance.

The time taken for all MAZE tests by the 1 mg/kg GEN group in the normal state was shorter compared to the vehicle group, and this difference was significant ($P < 0.05$) on day 1 of MAZE (A) (Fig. 6A). In the 10 mg/kg GEN group, the time was significantly shorter than the vehicle group in MAZE (A) (day 1 and day 3: $P < 0.01$; day 2: $P < 0.05$), but performance weakened as mazes were advanced (Fig. 6A). These results indicate that both 1 mg/kg and 10 mg/kg GEN improved spatial learning and memory, but only in the early stages of testing in normal male rats. In contrast, for the glucose-loaded group, the time taken by both GEN treated groups was significantly shorter than the vehicle group for MAZE (B) ($P < 0.05$) and (C) ($P < 0.01$, 0.05) (Fig. 6C). These findings indicate that both doses of GEN improved spatial learning and memory over time, when in a higher blood glucose state. It has been reported that GEN has ameliorating effects on brain function, in a number of disease models and in OVX rats [5–9,23]. The data reported here suggest that GEN is underworked when the body is in a normal state, but might lead to improved spatial learning and memory when homeostasis is lost. It has been reported previously that GEN decreased lipid peroxidation in the liver of STZ-induced diabetic rats [14]. Moreover, oxidative stress in the brain was decreased by oral administration of GEN in OVX rats [30]. In the context of glucose transporter proteins (GLUT), which introduce glucose uptake into the CNS [31], it has been found that GEN can increase GLUT-4 levels in regions of the cerebral cortex, in aged OVX rats, when under a state of decreased insulin sensitivity and impaired cerebral glucose homeostasis [32]. GEN might therefore result in improved spatial learning and memory by decreasing oxidative stress or regulating glucose homeostasis in the brain, when in an elevated blood glucose state, at the point of memory consolidation.

In the retention trials of the step-through passive avoidance test, the latency was not decreased by the glucose load (Fig. 7B). It had been reported that an elevation of blood glucose level affected on the spatial learning and memory which associated with hippocampus [28,33]. In the step-through passive avoidance test, it has been indicated that not only hippocampus but also another brain regions (e.g. amygdala [34–36]) were involved in that learning. From these factors, it was possible that the transient elevation of blood glucose level affected to the MAZE test, but did not affect to the step-through passive avoidance test.

Although significant differences were not observed, the 1 mg/kg GEN groups exhibited impaired memory performance compared to the vehicle group in both states, but most particularly the glucose-loaded group (Fig. 7A, B). We have previously reported that perinatal exposure to 1 mg/kg of GEN inhibits aversive learning and memory using the step-through passive avoidance test, in offspring rats [25]. We therefore presume that GEN inhibits aversive memory when the brain is more sensitive, such as a period of brain development or in higher glucose level state.

The results from the blood glucose levels showed no significant changes between groups in fasting blood glucose level. However, in the glucose load test, the blood glucose level 30 min following glucose load increased significantly from the fasting state, in both the vehicle and 10 mg/kg GEN groups, whereas a significant difference was not observed in the 1 mg/kg GEN group (Fig. 8). This observation suggests that a low dose of GEN controls the elevation of blood glucose level. A previous report suggested that GEN treatment prevents insulin resistance in aged OVX rats [8]. Furthermore, it has been reported that GEN stimulates insulin secretion in pancreatic ß-cells and promotes glucose uptake in L6 myotubes [16,37,38]. These factors could affect the regulation of blood glucose level due to GEN treatment in the present study. To regulate the blood glucose level after glucose load, 1 mg/kg of GEN was more effective than 10 mg/kg of GEN (Fig. 8). Previous study reported that an ER-ß selective agonist regulated the blood glucose level in a model of diabetes [39]. It has been shown that GEN has higher binding affinity to ER-ß than ER-α, and phytoestrogen showed non-monotonic dose response [1,7,40]. If the GEN affected to the blood glucose level through the estrogenic pathway, it might be possible that the low dose of GEN was more effective to regulate the blood glucose level.

In glucose-loaded rats, 1 mg/kg of GEN group showed improved the spatial learning and memory (Fig. 6C). However, 1 mg/kg GEN group in normal rats also showed ability to improve the spatial learning and memory without glucose load (Fig. 6A). It was possible that the effect of GEN appeared more clearly by the glucose load test. Only 1

mg/kg of GEN inhibited the elevation of blood glucose level (Fig. 8), whereas both doses of GEN improved the spatial learning and memory in glucose-loaded rats (Fig. 6C). Concerning this point, degree of elevation in blood glucose level might be related. Increase rate of blood glucose level at 30 min after the glucose load from 0 min were 28.7 %, 7.4 % and 17.4 %, for the vehicle group, 1 mg/kg GEN group, and 10 mg/kg GEN group, respectively. The improvement of spatial learning performance might come out by regulating the elevation of blood glucose level more than a certain level.

In conclusion, oral administration of GEN improved spatial learning and memory, but only in the early stages of testing in normal state male rats. In contrast, spatial learning and memory was improved by GEN treatment in an elevated blood glucose state at the time of memory consolidation.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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