Perinatal exposure to genistein, a soy phytoestrogen, improves spatial learning and memory but impairs passive avoidance learning and memory in offspring.

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Abstract

This study investigated the effects of perinatal genistein (GEN) exposure on the central nervous system of rat offspring. Pregnant dams orally received GEN (1 or 10 mg/kg/day) or vehicle (1 ml/kg/day) from gestation day 10 to postnatal day 14. In order to assess the effects of GEN on rat offspring, we used a battery of behavioral tests, including the open-field, elevated plus-maze, MAZE and step-through passive avoidance tests. MAZE test is an appetite-motivation test, and we used this mainly for assessing spatial learning and memory. In the MAZE test, GEN groups exhibited shorter latency from start to goal than the vehicle-treated group in both sexes. On the other hand, performances in the step-through passive avoidance test were non-monotonically inhibited by GEN in both sexes, and a significant difference was observed in low dose of the GEN-treated group compared to the vehicle-treated group in female rats. Furthermore, we found that perinatal exposure to GEN did not significantly alter locomotor activity or emotionality assessed by the open-field and elevated-plus maze tests. These results suggest that perinatal exposure to GEN improved spatial learning and memory of rat offspring, but impaired their passive avoidance learning and memory.

Keywords: Genistein, Isoflavone, Central nervous system, Perinatal exposure, Behavior, Non-monotonic dose response

1. Introduction

Isoflavone glycosides are found in soy beans, and include genistin, daidzin, and glycitin, which are mainly hydrolyzed by intestinal bacteria after oral intake. Following hydrolysis, these isoflavones become aglycones that correspond to genistein, daidzein, and glycitein, respectively, and can exert physiological effects [1]. Genistein (GEN) is one of the major types of phytoestrogens that interact with the estrogen receptor (ER) [2, 3], having higher binding affinity to ER than other phytoestrogens *in vitro* [4-6].

In Asian countries, especially Japan, soy foods are consumed in large quantities. Additionally, some infants are brought up on soy formulas due to cow milk allergies or lactose intolerance [7]. In cases such as these, many individuals, ranging from infants to adults, are exposed daily to isoflavones in their diets.

It has been shown that orally ingested GEN can cross the rat placenta and reach fetal brains from maternal serum [8]. In fact, one study detected GEN in the plasma of human umbilical cords, amniotic fluid, and in neonates, with concentrations that were similar to those in the maternal plasma [9]. These reports indicate that maternal GEN can cross the placenta and affect the central nervous system (CNS) of fetuses. Furthermore, the absorption and elimination of soy isoflavones in infants differs from adults since intestinal bacterial flora remains undeveloped in infants [10]. Due to the high exposure of soy in some fetuses and infants, it is important to define the effects of GEN on perinatal exposure.

The effects of perinatal or neonatal exposure to GEN on the reproductive system have been well documented. For example, male rats exposed to GEN during pre- or post-natal periods have been reported to exhibit demasculization or decreased reproductive behavior in adulthood [11, 12]. Additionally, perinatal or neonatal exposure to GEN in female rats, has been shown to cause irregular estrous cyclicity and histological changes in the ovaries, uterus, and mammary glands [13-16].

In contrast to its detrimental effects on reproduction, previous studies have also focused on the benefits of GEN on brain function in various adult animal models such as Alzheimer's disease [17, 18], global cerebral ischemia [19], and ovariectomy [20, 21]. However, only a few studies have examined the effects of GEN on learning and memory in rat pups [22]. Therefore, in the present study, we investigated the consequences of perinatal exposure to GEN on the CNS of offspring rats, particularly in relation to learning and memory.

2. Materials and Methods

2.1. Animals and treatments

Pregnant Sprague-Dawley rats at gestation day 6 (GD 6) were purchased from Kyudo Corp. (Saga). All animals were maintained in 12:12-h light-dark cycles (lights on from 07:00 to 19:00) at 22 ± 2°C and 55 ± 10% humidity.

Dams were divided into three groups: vehicle (n = 6), which received 0.5% Carboxymethyl Cellulose Sodium Salt (CMC-Na; Wako Pure Chemical Industries, LTD., Osaka) and GEN (LKT Laboratories, Inc., Minnesota, USA) groups, which received 1 mg/kg/day (n = 5) or 10 mg/kg/day (n = 6) of GEN dissolved in 0.5% CMC-Na. The administration of drugs to pregnant dams started from GD 10 to postnatal day (PND) 14. Oral administrations were conducted at the rate of 1 ml/kg/day under light anesthesia with halothane (Fluothane, Takeda Pharmaceutical Co. Ltd., Tokyo).

Pregnant rats were given free access to food and water during the experiment, and singly housed from GD 17. After birth, rat pups were culled to nine or 10 per litter on PND 3, and weaned on PND 20. After four weeks of age, pups were housed with a same-sex sibling. At 6-weeks-old, rats were separated into groups for either MAZE testing (an appetitive motivation test) or other behavioral tests (open-field, elevated plus-maze and step-through passive avoidance tests). At that time, animals which were assigned for MAZE testing were started on a light food-restricted diet to increase motivation for rewards.

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.

2.2. Open-field test

We used the open-field test to estimate locomotor activity and emotionality of rats as described by Hall [23]. The open-field apparatus was shaped like a bucket and consisted of a 60 cm in diameter bottom and enclosed by walls that were 50 cm in height. The floor of the apparatus was divided by black lines into 19 equal regions, and we illuminated the area using a light (100 W) placed 80 cm above the floor. The open-field contained an inner ring (placed 12–30 cm from the wall) and an outer ring (placed 0–12 cm from the wall). At the start of the experiment, rats were placed on

the center of the floor and the following parameters were measured for 3 min: ambulations (total number of crossing black lines on the floor), inner (number of crossing black lines of the inner circle), and rearing (number of times the rat stood up on its two hind-legs). Observations were conducted three times with two hours of intervals. The parameters of ambulation, inner, and rearing corresponded to indexes of locomotor activity, emotionality, and exploratory behavior, respectively. Open-field test conducted at 7 weeks of age.

2.3. Elevated plus-maze test

We used the elevated-plus maze test [24] as an estimate of anxiety. This maze was placed 60 cm above the floor, and was the form of a plus which had two closed arms (surrounded by walls 60 cm in height) and two open arms (no wall). Each arm (50 × 10 cm) was connected by a neutral zone (14 × 14 cm) which was placed in the center of the apparatus. At the beginning of each trial, rats were put in the neutral zone facing an open arm. The total number and total time spent in each of the closed and open arms were then measured for 5 min. The elevated-plus maze test conducted at 7 weeks of age.

2.4. MAZE test

We used the MAZE test as an estimate of spatial learning and memory as described before [25]. The MAZE apparatus was painted white and consisted of a large compartment (90 × 90 × 50 cm) with an attached goal partition (15 × 15 × 50 cm). The inside of the apparatus was illuminated by three bulbs (100-W) from 100 cm above the floor, and four different types of marks were attached to the wall surface. We made three levels of apparatuses in the big compartment by combining divider plates of various sizes (50 × 15 cm, 50 × 30 cm, 50 × 45 cm, 50 × 60 cm). The difficulty of MAZE was increased as the mazes were advanced [MAZE (A) → MAZE (B) → MAZE (C)] (Fig. 1). Three-hundred microliters of milk was used as the reward, and was placed in the goal partition. Experimenters wiped the entire MAZE apparatus after each trial in order to remove odors.

The MAZE procedure involved three different steps, carried out in the following order: Habituation → Training → Testing.

The “Habituation” step, performed for three consecutive days, was used as a way to familiarize rats to the apparatus and rewards.

After habituation, animals learned correct paths to the goal compartment by "Training" on the day before the "Testing" of each maze apparatus. In the training session, we used an apparatus that block off incorrect ways of the maze. At the beginning of the training trial, a rat was placed on the start point, and allowed to find the milk reward. Experimenters measured the time from start to successful attainment of the reward for each of three consecutive trials. If the rat could not reach the goal within 3 min, experimenters guided the rat to the goal. After each trial, the rat was removed from the maze and placed in their cage where they rested for 1 min. Following this brief rest, the rat was then placed back in the maze apparatus where they began the next trial.

The day following the "Training" stage, rats were evaluated for their spatial learning ability by "Testing" for three consecutive days. Testing was carried out in the same way as training in that animals were measured for their time and error for 5 min. Time was defined as the latency from start to attaining the reward, while error was defined as the number of approaches to incorrect areas. MAZE (A) was performed at 8 weeks, MAZE (B) was performed at 10 weeks, and MAZE (C) was performed at 12 weeks of age.

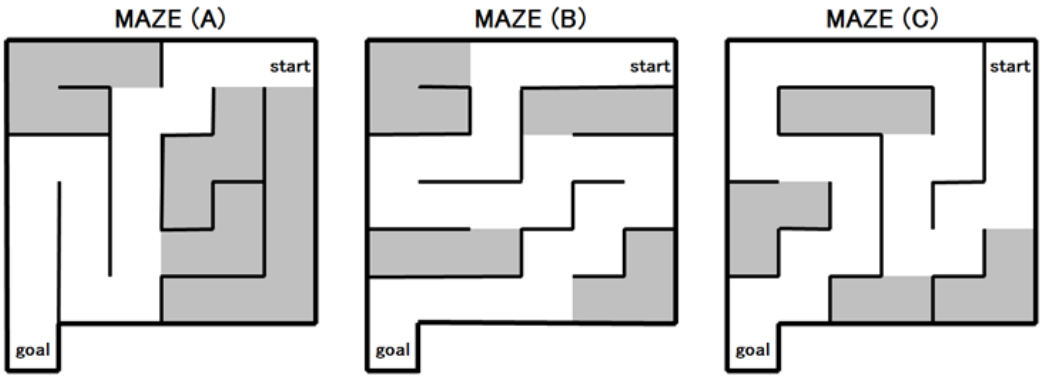

Fig. 1

2.5. Step-through passive avoidance test

We used the step-through apparatus to estimate passive avoidance learning and memory [26]. This apparatus (Shintecno Co. Ltd., Fukuoka) consisted of two chambers, a light chamber (10 × 20 × 12 cm) which was illuminated, and a dark

chamber (30 × 30 × 30 cm) which remained dark. These chambers were connected with a path (8 × 8 cm) and separated by a guillotine door. The dark chamber had a grid floor which elicited an electric current.

The step-through test consisted of three steps, and was conducted for three consecutive days at 11 weeks of age. On Day 1, each rat was habituated to the step-through apparatus. At the beginning of the habituation trial, the rat was placed in the light chamber for 10 s. The pathway between chambers was then opened, and the rat was allowed to move freely between the chambers for 90 s. On Day 2, each rat was placed in the light chamber, and the rat received a foot shock (1 mA) for 5 s using a shock generator (MSG-001, Toyo Sangyo Co. Ltd., Toyama) after first entering into the dark chamber. Each rat was measured for its latency to first enter into the dark chamber (acquisition time). On Day 3, each rat was placed in the light chamber, and latency to enter the dark chamber was again measured (retention time). In this step, the maximum of allotted measurement time was 300 s.

2.6. Statistical analysis

All results of the behavioral tests were analyzed using a two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests (Stat View, SAS, Cary, NC, USA). If the analysis showed $P < 0.05$, we considered that the results differed significantly between groups. All data are presented as means ± SEM.

3. Results

3.1. Reproductive ability

In the vehicle-treated group and 1 mg/kg GEN-treated group, all mother rats were able to give birth (Table 1). However, two of the six dams treated with 10 mg/kg GEN did not demonstrate any weight gain approximately one week from the start of administration (data not shown), and were not able to deliver pups (Table 1).

Each group exhibited a range in the number of pups they delivered. This range was found to be from nine to 16 in the vehicle-treated group, 10 to 15 in the 1 mg/kg GEN-treated group, and nine to 15 in 10 mg/kg GEN-treated group. No significant alterations were observed due to GEN exposure concerning mean litter size (Table 1).

Table 1

Reproductive ability of mother rats.

treated group	total treated dams	dams which were able to give birth	total pups in PND 3	mean litter size
vehicle	6	6	71	11.8
1 mg/kg GEN	5	5	62	12.4
10 mg/kg GEN	6	4	48	12.0

Table 1

3.2. Open-field test

In male rats, there were no significant differences observed between vehicle and GEN-treated groups in all parameters of the open-field test. However, the amount of ambulation gradually decreased over time in the 10 mg/kg GEN-treated group, and a significant difference ($P < 0.05$) was observed at 4 h between the 1 mg/kg GEN-treated group and the 10 mg/kg GEN-treated group (Fig. 2A). For the inner parameter, all groups showed similar values, except for the inner value scored at 2 h (Fig. 2B). Rearing in the 1 mg/kg GEN-treated group decreased slightly compared to the vehicle group at all measured time-points, but these changes were not significant (Fig. 2C).

In female rats, no significant differences were observed between groups for all parameters of the open-field test. In the 10 mg/kg GEN-treated group, the frequency of ambulation and inner instances were decreased when compared to the vehicle-treated group, however, these differences were not significant (Fig. 2D, E). Rearing behaviors were also not greatly altered by GEN treatments (Fig. 2F).

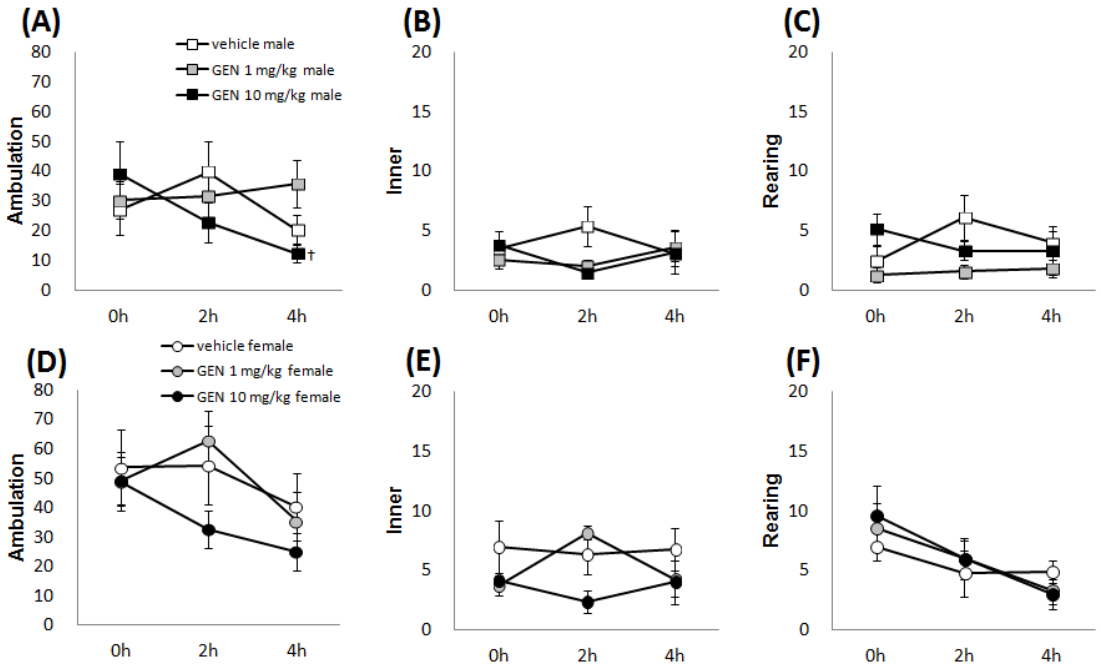

Fig. 2

3.3. Elevated plus-maze test

In male rats, significant differences were not observed between groups in all parameters of the elevated plus-maze test. The number of open arm entries decreased slightly in the 10 mg/kg GEN-treated group compared to the vehicle-treated group, while in the 1 mg/kg GEN-treated group, the number of closed arm entries slightly increased when compared to the vehicle-treated group. However, these slight differences were not significant (Fig. 3A). All groups spent similar amounts of time in both arms (Fig. 3C).

In female rats, the 1 mg/kg GEN-treated group showed fewer numbers of open arm entries than the vehicle-treated group, however, this difference was not significant (Fig.3B). The 10 mg/kg GEN-treated group spent slightly longer times in the open arms and slightly shorter times in closed arms compared to other treatment groups, however, these differences were not statistically significant (Fig. 3D).

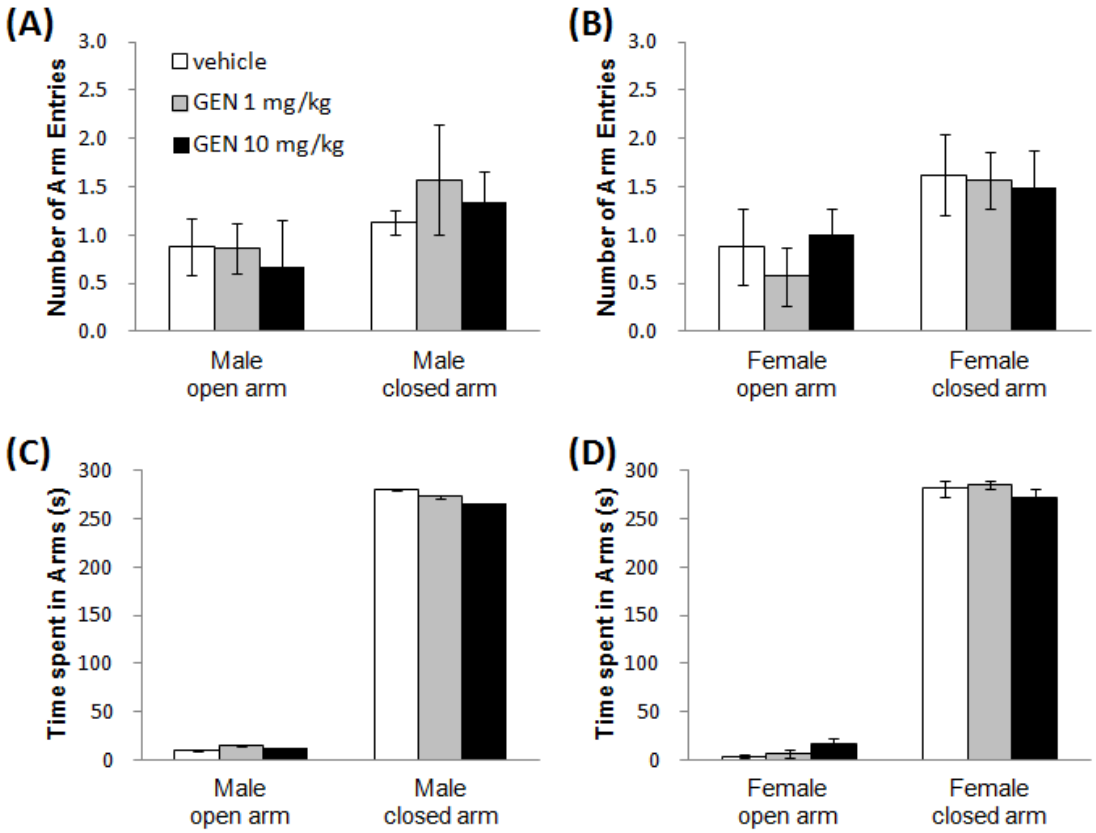

Fig. 3

3.4. MAZE test

In male rats, the vehicle-treated group showed a smooth learning curve over time (Fig. 4A), while rats in the 1 mg/kg GEN-treated group exhibited shorter latencies in all MAZE tests, with significant differences being observed at Days 1 and 2 of MAZE (A) ($P < 0.01$) and Days 1 and 3 of MAZE (B) ($P < 0.05$) (Fig. 4A). The 10 mg/kg GEN-treated group exhibited decreased times compared to the vehicle-treated group with the exception of Day 1 of MAZE (C), and significant differences were observed at Days 1 and 2 of MAZE (A) ($P < 0.01$, 0.05) and Day 2 of MAZE (B) ($P < 0.05$) (Fig. 4A). Although the 1 mg/kg GEN-treated group displayed a fewer number of errors than the vehicle-treated group in MAZE (A) and (B), the differences were not significant (Fig. 4B). However, in the 10 mg/kg GEN-treated group, errors gradually increased in MAZE (A), and these differences were significant in Day 3 ($P < 0.01$) compared to

the1 mg/kg GEN-treated group (Fig. 4B). There were no significant differences between groups in the frequency of errors made in MAZE (B) and (C) (Fig. 4B).

In female rats, the times of 1 mg/kg GEN-treated group were significantly shorter than the vehicle-treated group ($P < 0.01$, 0.05) in each day of MAZE (A), (B), and (C) (Fig. 4C). In the 10 mg/kg GEN-treated group, times were shortened compared to the vehicle-treated group in all days of MAZE (B), and in Days 2 and 3 of MAZE (C). These reduced times were significantly different at Day 3 of MAZE (B) (Fig. 4C). Significant differences were also observed between the 1 mg/kg GEN-treated group and 10 mg/kg GEN-treated group in all MAZE tests ($P < 0.01$, 0.05) (Fig. 4C). Furthermore, the number of errors made in the 1 mg/kg GEN-treated group decreased compared to the vehicle-treated group in MAZE (A) and (B), and significant differences were observed in Day 2 of MAZE (A) ($P < 0.05$) and Day 3 of MAZE (B) ($P < 0.01$) (Fig. 4D). The 10 mg/kg GEN-treated group showed fewer errors than the vehicle-treated group in MAZE (A), and a significant difference was observed in Day 2 ($P < 0.05$) (Fig. 4D). Finally, at Days 2 and 3 of MAZE (B), there were significant differences ($P < 0.05$) between the 1 mg/kg GEN-treated group and 10 mg/kg GEN-treated group (Fig. 4D), while there were no significant differences in the number of errors made in MAZE (C) for any of the groups (Fig. 4D).

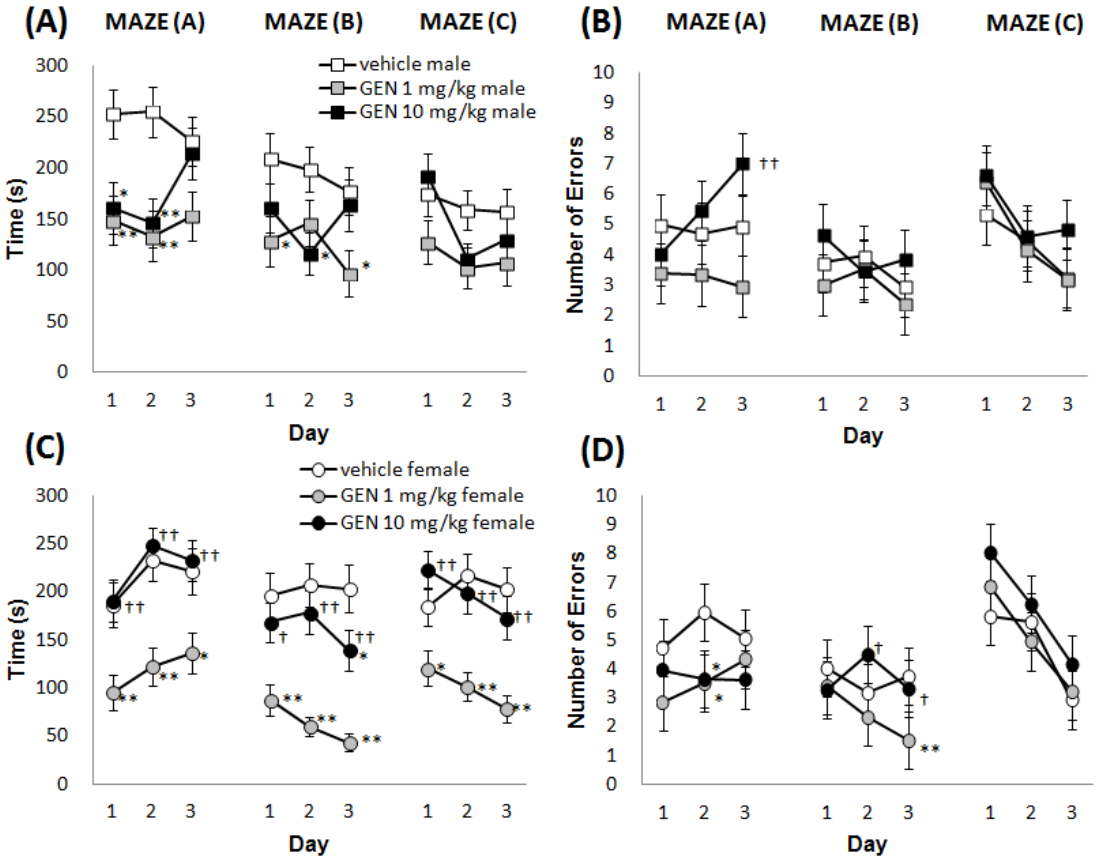

Fig. 4

3.5. Step-through passive avoidance test

In the training session, there were no significant differences between groups in male and female rats. However, during the retention trial, latencies were decreased by the perinatal exposures to GEN in both sexes (Fig. 5A, B), with female rats exposed to 1 mg/kg of GEN exhibiting significantly shorter latencies ($P < 0.05$) than the vehicle-treated group (Fig. 5B).

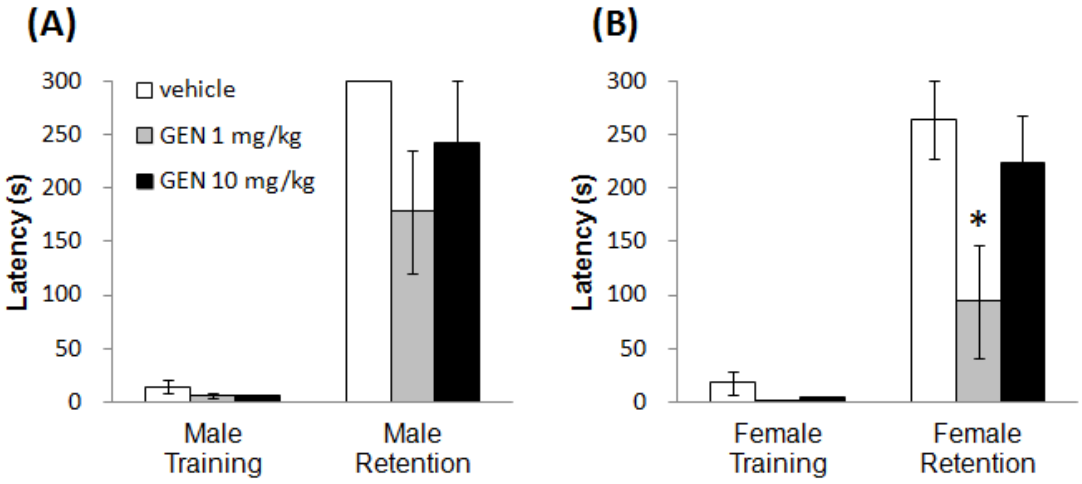

Fig. 5

4. Discussion

In the present study, we investigated the effects of perinatal exposure to GEN on the CNS in offspring rats using a battery of behavioral tests. Oral administrations of GEN to dams were conducted from GD 10 and extended to PND 14 to include the critical period for brain development in the rat pups.

In this study, we used GEN dosages of 1 mg/kg/day and 10 mg/kg/day. The 1 mg/kg/day of GEN seemed to match closest with physiologically relevant values as far as what individuals are exposed to in regions where soy is consumed in high quantities, such as Japan [27, 28]. The dosage of 10 mg/kg/day has been reported to improve brain function in adult disease models [17, 18, 29, 30].

In the current study, we found the impairment in the reproductive ability of two of the six dams that received 10 mg/kg/day of GEN (Table 1). There have been reports which suggest infertility in adulthood could be caused by neonatal exposure to GEN [31, 32] . In these reports, GEN had a dose-dependent inhibition on fertility, with half of the mice treated with 25 mg/kg of GEN being able to deliver live pups while none being treated with 50 mg/kg were able to deliver live pups. Our results showed that 10 mg/kg/day of GEN had some effects on a sustention of pregnancy. Thus the intake of large amounts of GEN requires careful attention, especially for pregnant women.

In the open-field test, although a significant difference in ambulation was observed between the 1 mg/kg GEN group and the 10 mg/kg GEN group in male rats ($P < 0.05$;

Fig. 2A), significant alterations by GEN were not observed in all parameters when we compared the GEN groups to the vehicle group in both sexes (Fig. 2A-F). In the elevated-plus maze test, GEN groups did not show any significant differences compared to the vehicle group (Fig. 3A-D). Thus, this study showed that perinatal exposure of GEN did not affect locomotor activity or emotionality. With regard to emotionality, a previous report suggested that GEN from soy-based diets could mitigate anxiogenic-induced effects of developmental exposure to Bisphenol A (BPA) [33]. Although there was little effect of GEN itself on anxiety in our study, it could be that GEN has the ability to relieve anxiety from an already anxiogenic condition.

We also investigated the effects of perinatal exposure to GEN on spatial learning and memory using the MAZE test. We found that in male rats, time in the 1 mg/kg GEN group decreased significantly compared to the vehicle group in each MAZE test (Fig. 4A). Additionally, we also observed a shortened time in the 10 mg/kg GEN group, however, these performances were inferior when compared to the 1 mg/kg GEN group (Fig. 4A). From these results, we can conclude that both dosages of GEN improved spatial learning and memory, but that 1 mg/kg/day of GEN was more effective than 10 mg/kg/day of GEN in male rats. In female rats, the times of the 1 mg/kg GEN group were significantly shorter than times recorded for the vehicle group, with significant differences being shown in all days of MAZE testing (Fig. 4C). Errors in the female 1 mg/kg GEN group also decreased compared to the vehicle group (Fig.4D). In the 10 mg/kg GEN group, however, times recorded were closely matched to the vehicle group than the 1 mg/kg GEN group (Fig. 4C). Errors recorded for the higher concentration GEN group were also fewer than those recorded for the vehicle group, but this reduction was only observed at MAZE (A) (Fig. 4D). These results indicate that perinatal exposure to 10 mg/kg/day of GEN exerted limited effects when compared to 1 mg/kg/day of GEN in female rats. As far as spatial learning and memory, we found that perinatal exposure to GEN improved these behaviors as assessed by the MAZE test, non-monotonically in both male and female offspring rats. Many studies have reported the non-monotonic dose-response of environmental endocrine disruptors such as BPA and diethylstilbestrol (DES) [25, 34]. Additionally, it has been suggested that phytoestrogens exert weak estrogenic effects at low concentrations and antiestrogenic effects at high concentrations [21, 35]. Therefore, it could be that high doses of GEN may weaken the beneficial effects of GEN through its actions on the estrogen pathway.

Interestingly, Ball et al. reported that perinatal exposure to GEN inhibited learning and memory when assessed by the Morris water maze [22]. The conflict in

results between our study and the findings reported by Ball et al. may be due to different learning mechanisms. The water maze is a learning paradigm characterized by a motivation to escape from water, whereas the MAZE test used in the current study is a learning paradigm characterized by motivation to receive a reward. It has been reported that appetitive long-term memory (LTM) is consolidated more quickly than aversive LTM [36, 37]. Moreover, Hirano et al. showed that different LTM systems are utilized in the hungry state [38]. The rats used for MAZE testing in this study were kept at a mildly hungry state, thus different effects from GEN exposure may have occurred.

Several factors could explain the ameliorating effects of perinatal GEN that we observed on spatial learning and memory in rat offspring. For example, one previous report showed that GEN could enhance nerve growth factor (NGF)-induced neurite outgrowth *in vitro* [39]. Therefore, perinatal exposure to GEN during the critical period for brain development might induce morphological changes in the nerves of rat offspring. Another potential factor that could explain the effects of GEN is via its activation of the estrogen pathway. It has been suggested that GEN has relatively high affinity for not only ER β but also G protein-coupled ER. Moreover, GEN can induce rapid increases in c-fos through a GRP30-mediated mechanism, and c-fos upregulation has been linked to neural plasticity [5, 40, 41]. Alternatively, it could be that a combination of these factors is what is responsible for producing the beneficial effects of perinatal exposure to GEN.

In the current study, we observed that the effects of perinatal GEN exerted slight differences on learning and memory when comparing males and females. A previous report has suggested that the effect of GEN is affected by a number of factors such as the concentration of phytoestrogens administered, the concentration of endogenous estrogens, gender, and menopausal status [42]. An ovariectomy was not conducted for female rats in the present study in order to reveal the effect of GEN exposure to females without any surgery. Therefore, we are unable to exclude the intervention of endogenous estrogen in our findings, so we cannot directly compare the results of males and females.

In this study, GEN improved the spatial learning and memory in both sexes, but impaired passive avoidance learning, especially in female rats. One possible reason for this phenomenon is the different effects of GEN on various brain regions. For example, the MAZE test involves place learning which requires the hippocampus [43]. On the other hand, there have been some studies suggesting that the step-though passive avoidance test involves the striatum. Injection of atropine, an

acetylcholine-receptor blocker, into the caudate nucleus has been shown to impair performance on the passive avoidance [44]. Another study reported memory disruption in the avoidance task induced by stimulation to the caudate nucleus [45]. Considering these factors, our study suggests that GEN improved spatial learning and memory via its actions on the hippocampus, and inhibited learning and memory related to passive avoidance through involvement of the striatum. Our results support a previous study that was conducted on young adult female rats where acute GEN was shown to enhance place learning via its actions through the hippocampus and impaired response learning by involving the striatum [46].

In conclusion, the current study suggests that perinatal exposure to GEN improved spatial learning and memory assessed by MAZE test in both male and female rats, and inhibited passive avoidance learning and memory assessed by the step-through passive avoidance test especially in female rats. Studies such as these are extremely important because of the fact that metabolism of soy isoflavone in infants is different than metabolism in adults; therefore, it is important to get a complete understanding of the impacts of GEN intake during perinatal periods.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figure Legends

Fig. 1. MAZE apparatus. Three types of MAZE that gradually increased in difficulty were employed [MAZE (A) → MAZE (B) → MAZE (C)]. In these figures, white areas represent correct ways to the reward, while gray areas represent the incorrect paths.

Fig. 2. Open-field test. We used male (A, B, C) and female rats (D, E, F) for measurement of ambulation (A, D) to indicate total number of crossings, inner (B, E) as a parameter to measure the number of crossings inside the inner circle, and rearing (C, F) as the frequency of upright stances on hind-legs. Results are expressed as mean ± SEM. † $P < 0.05$ indicates a significant difference from the 1mg/kg GEN group.

Fig. 3. Elevated-plus maze test. We used male (A, C) and female rats (B, D) for measurement of the number of arm entries (A, B) and time spent (C, D) in each arm. The results are expressed as mean ± SEM.

Fig. 4. MAZE test. We used male (A, B) and female rats (C, D) for measurement of time (A, C) as the latency required to reach the goal of the milk reward, and error (B, D) as the number of entry to the error areas. Time and error were the average of three trials per each testing day. The results are expressed as mean ± SEM. *$P < 0.05$ and **$P < 0.01$ indicate significant differences from the vehicle group, and † $P < 0.05$ and ††$P < 0.01$ indicate significant differences from the 1 mg/kg GEN group.

Fig. 5. Step-through passive avoidance test. We used male (A) and female rats (B) to measure latency until rats first entered into the dark chamber. The results are expressed as mean ± SEM. *$P < 0.05$ indicates a significant difference from the vehicle group.