Inhibition of oestrogen biosynthesis induces mild anxiety in C57BL/6J ovariectomized female mice

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Abstract: Objective Letrozole, a next-generation aromatase inhibitor, has become a favored drug for the treatment of breast cancer in postmenopausal women. Although letrozole is generally well tolerated, its adverse effects on the central nervous system have been reported. The present study aimed to assess the behavioural outcomes of letrozole administration in mice to determine its side effects. **Methods** C57BL/6J female ovariectomized mice received administration of letrozole (2.5 mg/kg per day) or vehicle by gavage for 3 weeks. Behavioural tasks were used to assess anxiety, depression, as well as learning and memory in mice. **Results** Letrozole-treated mice showed an increased latency to enter the inner area of the chamber on the third day of the open field test, and traveled a shorter distance in the open arms of the elevated plus maze. No significant difference was found in the light-dark box or forced swimming task between letrozole-treated and vehicle-treated mice. Besides, letrozole did not change the spontaneous alternation behaviour of mice in the Y-maze. In the Morris water maze, mice administered with letrozole exhibited an improvement in spatial learning and memory compared with the vehicle-treated mice. **Conclusion** Our results indicate that the inhibition of oestrogen biosynthesis results in mild anxious behaviour, which may be a consideration in the treatment of breast cancer in postmenopausal women using aromatase inhibitors.

Keywords: anxiety; depression; aromatase inhibitor; learning and memory

1 Introduction

Breast cancer is the second leading cause of cancer death in women today. Among endocrine therapies, the third-generation aromatase inhibitors (AIs) offer a promising approach for treating hormone-sensitive breast cancer in postmenopausal women with higher specificity, greater efficacy, and less toxicity than previous treatments^[1–3]. However, AIs have been associated with higher incidences

of bone fracture^[4], skeletal and cardiac events^[3], gynaecologic changes and lipid metabolism disorders^[5]. Compared with these side effects, changes in the central nervous system induced by AIs have attracted less attention.

Aromatase, a key enzyme for oestrogen synthesis and a treatment target for breast cancer, is widely expressed in the central nervous system^[6–8], where it is classically associated with the regulation of reproduction^[9]. Recently, a growing body of evidence has indicated that aromatase activity is also involved in modulation of mood and affective status. Aromatase knockout female mice, but not male mice, show depressive-like behaviours^[10], and polymorphisms in the *CYP19* gene are associated with depressive

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symptoms in women^[11]. Furthermore, aromatase has also been confirmed to play an important role in regulating aggressive behaviour^[12,13]. Recently, emerging evidence has indicated that aromatase may regulate nonreproductive behaviour by modifying synaptic plasticity and function. Rune and colleagues have provided solid evidence indicating that the pharmacological inhibition of aromatase activity in hippocampal slices decreases the number of dendritic spines and the number of dendritic spine synapses^[14–16]. In addition, axon outgrowth in developing hippocampal neurons can be reduced by aromatase inhibition^[17]. Studies have also reported that reelin and GAP-43 may be involved in the regulation of synaptic plasticity by aromatase inhibition^[17,18].

However, only a few studies on endocrine therapy for breast cancer have focused on AI-induced changes in the central nervous system, including effects on cognition and mood disturbance^[19–24]. In postmenopausal breast cancer patients, aromatase inhibition therapy further decreases oestrogen production to the basal level^[25,26], which may have effects on mood and cognitive function. Therefore, investigating the side effect of AIs on the central nervous system will help to identify unknown biological effects of AIs, as well as to minimize the side effects and optimize endocrine-based therapy for patients. In the present study, a battery of behavioural tasks was employed to investigate whether locomotor activity, anxiety, learning and memory were influenced by AI administration in mice.

2 Materials and methods

2.1 Animals A total of 16 female C57BL/6J mice (6-week-old) were used in the study. The mice were group-housed ($n = 4$ in each cage) in polycarbonate cages containing woodchip shavings for bedding under a 12:12 h light/dark cycle (lights on at 7:00 am) at the temperature of (22 ± 1) °C. Food and water were available *ad libitum*. All

experiments were carried out in accordance with the Animal Care and Use Committee of the University of Science and Technology. All efforts were made to minimize animal suffering as well as to reduce the number of animals used.

2.2 Surgery, drug treatment and body weight measurement Ovariectomy was performed in 16 6-week-old mice by removing the bilateral ovaries under anaesthesia by 5% chloral hydrate (5 mL/kg body weight). After 2-week recovery, the ovariectomized (OVX) mice were divided into 2 groups. In the first group, mice ($n = 8$) were treated with letrozole (Femara; Novartis) by gavage at a dose of 2.5 mg/kg per day, according to the previous description^[27], for 3 successive weeks. In the other group, mice ($n = 8$) received vehicle treatment. The letrozole tablets were diluted in physiological saline. Previous studies have shown that systemic administration of letrozole selectively depletes serum estradiol^[25,28]. Evidence from both humans and laboratory animals suggests that oestrogen plays an important role in the regulation of body weight^[29,30], thus here the body weight was measured at each weekend during drug treatment.

2.3 Behavioural tests All behavioural tests were performed during the light phase of cycle in a new room similar to the housing room. Before each test, the animals were given 20 min for adaptation to the testing environment. The timetable of these tests was shown in Fig. 1. All the tasks were monitored by a video camera.

2.3.1 Open field test To detect spontaneous locomotor activity and anxiety, an open field procedure was designed according to the previous description^[31] with minor modifications. An open, wooden box (50 cm × 50 cm × 25 cm) with smooth surface was used in the study. For each trial, an animal was placed into one of the 4 corners, facing the corner, and was permitted to explore the environment *ad libitum*. Activity was measured for 5 min for 3 successive

Fig. 1 A timeline of the behavioural tests. OF: open field; LDB: light-dark box; EPM: elevated plus maze; MWM: Morris water maze; FS: forced swimming.

days. The latency to the inner area, the duration and the distance traveled in the inner area were recorded.

2.3.2 Light-dark box (LDB) test The LDB test is a validated task for assessing anxiety in rodents^[32]. It consisted of 2 compartments: one light box and one dark box, of the same size (25 cm × 25 cm × 25 cm), separated by a Plexiglas panel. An arch-shaped hole was made in the bottom of the panel to allow passage of the animal. In each trial, a mouse was gently placed in the light compartment facing the wall opposite to the hole, and was allowed to explore the LDB for 5 min. The latency to enter the dark box, the number of transition to the dark box, and the duration in the dark box were recorded.

2.3.3 Elevated plus maze (EPM) test The EPM test was designed according to the description of Walf *et al.*^[33]. Briefly, the maze (made of Plexiglas) consisted of a plus-shaped apparatus, with 2 opposite closed arms (30 cm × 6 cm) enclosed with walls (15 cm in height) and 2 opposite open arms (30 cm × 6 cm, without walls). The whole apparatus had a central arena (6 cm × 6 cm) and was elevated 80 cm above the floor. Each mouse was placed in the central arena of the maze facing an open arm and allowed to explore the maze for 5 min. The duration and distance traveled in the open arms and the frequency of entering the open arms were analyzed.

2.3.4 Y-maze test Spontaneous alternation behaviours were assessed by the Y-maze according to the previous study^[34], with some modifications. The apparatus consisted of 3 Plexiglas arms (45 cm × 15 cm × 30 cm) defined as the placing arm, the novel arm and the familiar arm. The Y-maze task included 2 sessions conducted on successive days. In the first session, the novel arm was closed, and mice were placed at the end of the placing arm facing the wall and allowed to explore freely in the maze for 15 min. Twenty-four hours later, the second session was performed. The novel arm was opened, and the mice traveled freely within the 3 arms for 5 min. The total locomotor activity was recorded using a video camera. The distance or duration in the novel arm was recorded and calculated as the ratio to total distance or duration in novel and familiar arms [Novel / (Novel + Familiar)].

2.3.5 Morris water maze (MWM) test The MWM test was used to test spatial learning and memory^[31]. The pool (1.2 m in diameter) was filled with opaque water and surrounded by complex maze cues. The escape platform (10 cm in diameter) was placed in the center of a designated quadrant with its top being 1 cm below the water surface. In the hidden-platform test, mice received 4 trials per day for training for 6 d. A probe test was conducted on day 7. The starting point of each trial was designed according to the method of Vorhees and Williams^[35]. The mouse was allowed 60 s to find the platform, and stayed there for 30 s. If the mouse failed to find the platform within 60 s, it was guided to the platform and allowed to stay there for 30 s. During the probe test session, the hidden platform was removed from the pool, and the mouse was allowed to swim for 90 s. The latency to find the platform in the hidden-platform test and the duration in the target quadrant in the probe trial were analyzed.

2.3.6 Forced swimming (FS) test The FS test was carried out according to the method of Zhu *et al.*^[36], with some modifications. The behavioural cylinder (40 cm high and 25 cm in diameter) was filled with water to the height of 30 cm and maintained at 24–25 °C. The FS test paradigm included 2 sections: an initial 15-min pre-test and a following 5-min test, with a 24-h interval. After each test, the mouse was removed from the cylinder, dried with towels, and stayed in a heated cage for 15 min followed by its home cage. The total immobile time and swimming distance during the second 5-min test were recorded and analyzed. Mice were considered immobile when they did not make any active movements^[36].

2.4 Tissue preparation and oestrogen measurement

Two days after the behaviour tasks, the mice were anesthetized with 5% chloral hydrate (5 mL/kg body weight) and decapitated. Blood and hippocampal samples were prepared for 17 β -estradiol measurement. Blood was collected in tubes containing heparin sodium as an anticoagulant and centrifuged at 6 000 rpm for 10 min at 4 °C. The plasma was collected and stored at -70 °C until use. Mouse 17 β -estradiol level was detected by the enzyme-linked immunosorbent assay (ELISA) kit (Biosource; Carlsbad, CA,

USA). To prepare 17β -estradiol samples of hippocampus tissue, the right hippocampus was separated, homogenized (15 passes in a Teflon-glass homogenizer; 600 RPM) in PBS and centrifuged at 12 000 rpm for 10 min at 4 °C. The supernatant was collected and stored at -70 °C for 17β -estradiol measurement. To determine the gross hippocampal protein, the precipitate was lysed in a cocktail containing RIPA buffer (Roche; Indianapolis, IN, USA) for 30 min and centrifuged at 12 000 rpm for 10 min at 4 °C. The supernatant was then collected and stored at -70 °C for gross protein measurement by the Lowry method. 17β -Estradiol concentration in the hippocampus was normalized to the gross protein.

2.5 Statistical analysis Data were presented as means $\pm$ SEM. The latency to find the platform in MWM was analyzed with repeated-measures ANOVA with one between-subject (LET treatment) factor and one within-subject (day) factor. Other data were analyzed by the independent-sample *t*-test. * $P < 0.05$ was indicated as statistically significant.

3 Results

3.1 Body weight The changes in body weight during drug treatment were shown in Fig. 2. Letrozole-treated mice gained significantly more weight than did the control subjects at the ends of week 1 ($P < 0.01$) and week 2 ($P < 0.001$).

3.2 Oestrogen concentration After completion of the behavioural tasks, oestrogen levels in the hippocampus and the plasma were measured. As shown in Fig. 3, hippocam-

Fig. 2 Body weight gain under letrozole and vehicle administration. Data were analyzed with independent-sample *t*-test. ** $P < 0.01$, *** $P < 0.001$. CON: control group. LET: letrozole treatment group.

pal level of 17β -estradiol was lower in letrozole-treated mice than in vehicle-treated mice ($P < 0.01$, Fig. 3A). However, there was no significant difference in plasma 17β -estradiol level between the 2 groups (Fig. 3B).

3.3 Behavioural task performance In the open field test, performance in the inner area usually indicates the anxiety level. In the present study, the latency of letrozole-treated mice to enter the inner area decreased significantly compared with that of vehicle-treated mice on the third day of the task (Fig. 4C, $P < 0.05$). Other measures, including the distance traveled and duration in the inner area, varied on each of the 3 days, but no significant difference was found between the 2 groups (Fig. 4B, D).

Letrozole-treated mice also showed anxious behaviour in the EPM task. Both the distance traveled in the open

Fig. 3 Oestrogen level in the hippocampus (A) and the plasma (B) in mice with letrozole or vehicle treatment. Data were represented as means $\pm$ SEM and analyzed with independent-sample *t*-test; ** $P < 0.01$. CON: control group. LET: letrozole treatment group.

Fig. 4 Results of the open field task. A: Representative activity tracers of letrozole- and vehicle-treated mice during 5 min of the first day task. The duration in the inner area (B), the latency to enter the inner area (C) and the distance traveled in the inner area (D) were analyzed. Data were presented as means $\pm$ SEM, and analyzed by independent-sample *t*-test. * $P < 0.05$. CON: control group. LET: letrozole treatment group.

Fig. 5 Results of the elevated plus maze test. A: Representative activity tracers of letrozole- and vehicle-treated mice during a 5-min sample period. The duration (B) and distance traveled (C) in the open arms and the frequency of entering the open arms (D) were analyzed. Data were presented as means $\pm$ SEM, and analyzed by independent-sample *t*-test. * $P < 0.05$. CON: control group. LET: letrozole treatment group.

arms (Fig. 5C, $P < 0.05$) and the frequency of choosing the open arms (Fig. 5D, $P < 0.05$) of letrozole-treated mice decreased compared with those of vehicle-treated mice. However, there was no significant difference in the duration in the open arms between the 2 groups (Fig. 5B).

However, the LDB task revealed no significant difference in the latency to enter the dark box (Fig. 6A), in the time spent in the dark box (Fig. 6B), or in the times of

transition (Fig. 6C), between letrozole-treated and vehicle-treated mice. Similarly, in the FS test, the immobile time (Fig. 7A) and the total swimming distance (Fig. 7B) of letrozole-treated mice did not differ from those of vehicle-treated mice, indicating no depression behaviour of letrozole-treated mice.

In addition, there was no significant difference in distance (Fig. 8A) or duration (Fig. 8B) in the novel arm

Fig. 6 Results of the light-dark box task. There was no significant difference in the latency to enter the dark box (A), in the time spent in the dark box (B) or in the times of light-dark box transition (C), between letrozole-treated and vehicle-treated mice. Data were presented as means $\pm$ SEM, and analyzed by independent-sample t -test. CON: control group. LET: letrozole treatment group.

Fig. 7 The effects of letrozole and vehicle administration on the total time of immobility (A) and total distance of swimming (B) in the forced swimming test. Data were presented as means $\pm$ SEM, and analyzed by independent-sample t -test. CON: control group. LET: letrozole treatment group.

Fig. 8 The effects of letrozole and vehicle administration on novel arm preference. There was no significant difference in distance (A) or duration (B) in the novel arm between letrozole-treated and vehicle-treated mice in the Y-maze task. Data were presented as means $\pm$ SEM, and analyzed by independent-sample t -test. CON: control group. LET: letrozole treatment group.

Fig. 9 Learning and memory in the hidden version of the Morris water maze. **A:** The latency to find the platform in the acquisition trials. Data were analyzed with repeated-measures ANOVA, between-subject (letrozole treatment) [$F(1,13) = 1233.28$, $P < 0.001$], within-subject (days) [$F(5,65) = 4.77$, $P = 0.001$]. **B:** The duration in the target quadrant during the probe trial. **C:** Representative activity tracers for letrozole- and vehicle-treated mice during the probe trial. CON: control group. LET: letrozole treatment group.

between letrozole-treated and vehicle-treated mice in the Y-maze task, indicating that letrozole treatment did not change the spontaneous alternation behaviour of OVX mice.

The MWM test was used for assessing spatial learning and reference memory (Fig. 9). Repeated-measures ANOVA revealed that both the letrozole-treated mice and the control mice showed improvements in spatial learning and memory across the day-to-day acquisition trial [$F(5,65) = 4.77$, $P = 0.001$]. Compared with control animals, letrozole-treated mice showed better performance in learning and memory in acquisition trials [$F(1,13) = 1233.28$, $P < 0.001$]. No statistical difference was found in the duration in the target quadrant during the probe trial, which was performed 24 h after the last acquisition trial (Fig. 9B).

4 Discussion

In the present study, we showed that systemic administration of letrozole, a widely used AI in clinical practice, for 3 weeks, induced mild anxiety-like behaviour in OVX female mice. Interestingly, the learning and memory capacity of letrozole-treated mice was also improved slightly by letrozole administration.

Because of the fluctuation of gonadal hormones during menopausal transition, there is a greater susceptibility to most types of anxiety/depression disorders and cogni-

tive impairment among women^[37-41]. For postmenopausal breast cancer patients, aromatase inhibition therapy further decreases oestrogen production to a basal level^[25,26], which may exacerbate the central nervous system dysfunction. Therefore, investigation of the side effects of AIs on the central nervous system is of great importance. The cognitive disorders in breast cancer patients receiving endocrine therapy have been studied. The BIG 1-98 randomized trial has found that breast cancer patients taking adjuvant letrozole during the fifth year of treatment have better cognitive functions than those taking tamoxifen^[42]. Cognitive impairment in postmenopausal breast cancer patients has recently attracted more attention^[19,21,24,43,44].

However, results of animal studies seem to be inconsistent with clinical findings on cognition function. Several animal studies have demonstrated that inhibition of 17β -estradiol by AIs does not impair but rather improves learning and memory in several tasks^[27,45,46]. Consistent with these findings, our results also showed that mice treated with letrozole showed improvements in spatial learning and memory earlier than vehicle-treated mice (Fig. 9A). The different outcomes between clinical studies and animal findings are possibly due to the class of memory tested: clinical trials focus on verbal and visual memory, while rodent studies primarily test spatial-related memory. A study of Cherrier *et al.* revealed that AIs can suppress verbal memory improvement but have no effect on spatial

memory progress induced by testosterone supplementation^[47].

However, compared with studies on cognition, mood disorders in breast cancer patients treated with AIs have been rarely examined. Anxiety and depression are among the most common disorders in cancer patients and are associated with reduced quality of life, abnormal illness behaviour and reduced responsiveness to endocrine therapies^[48,49]. In the present study, the anxiety and depression behaviours in mice after administration of AIs were measured. Four related tasks were selected: EPM test, open field test, LDB test and FS test. Behavioural outcomes showed that letrozole-treated mice traveled a shorter distance in the open arms of the EPM test (Fig. 5C, $P < 0.05$) and avoided the inner area of the open field (Fig. 4C, $P < 0.05$) compared with vehicle-treated mice. These results indicate that the mice receiving letrozole administration are in an early stage of anxiety. In addition, no significant difference was found in immobile time between the 2 groups in the FS test. Taken together, our results are consistent with clinical findings of changes of response to oestrogen inhibition^[20,22]. Studies in other mice models are also in line with the findings in the present study. Dalla *et al.* found that aromatase knock-out female mice, another 17 β -estradiol-deficient model, showed depressive-like behaviour^[10]. In natural aging, Walf *et al.* found that 17 β -estradiol replacement in old female subjects reduced anxiety-like and depression-like behaviours in both rats and mice^[50,51].

In addition, we also found that the 17 β -estradiol level in the hippocampus of letrozole-treated mice was lower than that of vehicle-treated subjects (Fig. 3A, $P < 0.01$). Accumulating evidence shows that the inhibition of oestrogen synthesis by AIs may modify synaptic plasticity in the hippocampus by interacting with several molecular and cellular processes. Rune and his colleagues reported that the numbers of dendritic spines, synapses, and synaptic proteins were significantly down-regulated in response to letrozole^[14,15] in a region-specific manner^[16]. Moreover, axon outgrowth in developing hippocampal neurons was reduced by aromatase inhibition^[17]. Emerging evidence

indicates that reelin and GAP-43 might be involved in the regulation of synaptic plasticity by letrozole administration^[17,18]. The changes occurring at the hippocampal synapse may have important implications for interpreting the behavioural outcomes induced by AIs administration.

In summary, our results show that the inhibition of oestrogen biosynthesis by AIs treatment for 3 weeks induces mild anxious behaviour in mice. Further investigations of the mechanism underlying the behavioural changes induced by AIs are required.

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抑制雌激素的生物合成会引起 C57BL/6J 卵巢摘除小鼠的轻微焦虑样行为

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摘要: 目的 来曲唑作为新一代芳香化酶抑制剂已广泛用于治疗妇女绝经后的乳腺癌。虽然来曲唑治疗具有良好的耐受性, 但其对中枢神经系统的副作用还需要更多的研究。方法 将卵巢摘除的C57BL/6J雌性小鼠每天给予来曲唑(2.5 mg/kg)或赋形剂灌胃处理, 共持续3周。给药结束后, 对小鼠自主运动、探索、焦虑、抑郁以及学习和记忆行为进行分析。结果 与对照小鼠相比, 来曲唑处理小鼠的体重显著增加, 海马组织匀浆中的雌激素水平显著降低。旷场实验中, 来曲唑处理小鼠在第3天任务中进入旷场中央区域的潜伏期显著增长。高架十字迷宫中, 来曲唑处理小鼠在开放臂上运动距离变短。在明暗箱、强迫游泳和Y-迷宫任务中, 来曲唑处理小鼠行为未有显著改变。在Morris水迷宫中, 来曲唑给药小鼠的空间学习和记忆能力有所提高。结论 系统性芳香化酶抑制剂降低了小鼠中枢神经系统中雌激素的含量, 并且导致了焦虑样行为的增加, 提示使用芳香化酶抑制剂治疗乳腺癌可能对中枢神经系统产生副作用。

关键词: 焦虑; 抑郁; 芳香化酶抑制剂; 学习和记忆