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Original Article

Lithium chloride alleviates cognitive impairment and decreases anxiety-like behavior in diabetic mice

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Abstract

Clinical and experimental studies have demonstrated that type 2 diabetes mellitus (T2DM) affects the brain structure and function, leading to cognitive impairments. Previous studies have reported putative neuroprotective effects of lithium in certain neurodegenerative disorders. However, the beneficial effects of lithium on improving cognitive impairments and alleviating affective disorders induced by T2DM remain to be elucidated. In the present study, we aimed to investigate the effects of lithium chloride treatment on cognitive and affective functions in diabetic mice. We found that the diabetic mice exhibited hyperglycemia and cognitive deficits in the novel object recognition test (NORT) and novel object location recognition test (NOLT), increasing anxiety-like behavior and depressive-like behavior. The treatment with lithium chloride normalized the blood glucose level, improved cognitive function in NOLT and alleviated anxiety-like behavior in diabetic mice. Our findings provide added information regarding the therapeutic benefits of lithium against diabetes-associated cognitive and affective impairments.

Keywords: lithium chloride, cognitive impairment, affective disorders, diabetes mellitus

1. Introduction

The global prevalence of diabetes is continuously rising. It is estimated that almost 285 million people are currently suffering from diabetes worldwide and its prevalence is estimated to reach 592 million people worldwide by 2035 (Guariguata *et al.*, 2014). Type 2 diabetes mellitus (T2DM) has been considered a common metabolic disorder that can cause many complications including cardiovascular disease, nephropathy, and retinopathy (Zheng, Ley, & Hu, 2018). A growing body of evidence indicates that T2DM is associated with mild cognitive impairment (MCI) and is a risk factor of dementia including Alzheimer's disease (AD) (Feinkohl, Price, Strachan, & Frier, 2015; Vagelatos & Eslick, 2013). Moreover, epidemiological studies have reported a link between diabetes mellitus and neuropsychiatric disorders such as depression (Khaledi, Haghighatdoost, Feizi, & Aminorroaya, 2019) and anxiety (Khuwaja *et al.*, 2010).

Neuroimaging studies in humans have demonstrated that patients with T2DM exhibit alterations in brain structures and functions that are associated with impairments in several cognitive domains (Biessels & Reijmer, 2014; Gu *et al.*, 2022; Xiong *et al.*, 2020). Experimental studies have also revealed that diabetes mellitus caused cognitive impairments accompanied by mitochondrial dysfunction, blood-brain barrier breakdown, Ca^{2+} signaling disruption, abnormal

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neuronal structure and function, and neurodegeneration (Biessels & Despa, 2018; Chen *et al.*, 2021). However, the exact molecular mechanisms underpinning the cognitive impairments induced by T2DM are not fully understood.

Lithium has been used for the treatment of bipolar disorder for a long time. More recently, lithium has also been regarded as a neuroprotective agent due to its effects on several mechanisms of neuronal homeostasis including the activation of neurotropic response, modulation of oxidative stress and inflammatory response, enhancement of mitochondrial function, and reduction of hyperphosphorylation of tau protein and amyloid beta 1-42 (Aβ1-42) production (Damri, Shemesh, & Agam, 2020; Forlenza, De-Paula, & Diniz, 2014). However, the beneficial effects of lithium on improving cognitive impairments and alleviating affective disorders induced by T2DM are unclear.

Therefore, in the present study, we aimed to investigate the effects of lithium chloride treatment on cognitive and affective functions in diabetic mice induced by the combination of a high-fat diet with an injection of streptozotocin. Our findings will solicitate the use of lithium in clinical implications for the treatment of cognitive dysfunction and mood disorders in patients with T2DM.

2. Materials and Methods

2.1 Generation of diabetic mice and drug treatment

Eight-week-old male C57BL/6N mice were obtained from Nomura Siam International, Thailand. The animals were housed in a climate-controlled environment with alternating 12h light–dark cycles. After one week of acclimatization, the animals were fed either a control diet (n=10) offering a total of 3.04 kcal/g (containing 4.5% crude fat, 24% crude protein) (National Animal Center, Salaya Campus, Mahidol University, Bangkok, Thailand) or a high-fat diet (n=20) supplemented with 25% sucrose (containing 14.4% crude fat, 24.3% crude protein) (Quick fat; CLEA, Japan) offering a total of 4.11 kcal/g for four weeks. The diabetic animal model was constructed by an intraperitoneal injection of streptozotocin (STZ), dissolved in cold citrate buffer (0.01 M, pH 4.5) at a dose of 100 mg/kg (Janthakhin, Kingtong, Aphibanthammakit, & Juntapremjit, 2023) with an injection volume of 10 mL/kg (Sigma-Aldrich), while the normal control group (NC) received the same volume injection of citrate buffer (vehicle). One week after the STZ injection, a fasting blood glucose (FBG) test was applied to assess the diabetic model. Mice that had a FBG higher than 200 mg/dL (Kang *et al.*, 2017) were included in the study. One mouse that did not meet the criterion was excluded from the study. The diabetic mice were then divided into two groups: the diabetic group (DM) and the LiCl-treated diabetic group (DM+LiCl). The LiCl-treated diabetic group received a daily intraperitoneal injection of lithium chloride (LiCl) at the dose of 100 mg/kg of the body weight for six weeks whereas the DM group received an injection of normal saline for the same duration. This dose of LiCl has been chosen as a previous study demonstrated its neuroprotective effects in intracerebroventricular streptozotocin induced memory deficit model (Ponce-Lopez, Liy-Salmeron, Hong, & Meneses, 2011). The behavioral tests were performed at six weeks after the drug treatment. The experimental protocol is shown in Figure 1. All experiments were guided following the Animal Care and Use Committee of Burapha University (IACUC Number 005/2565).

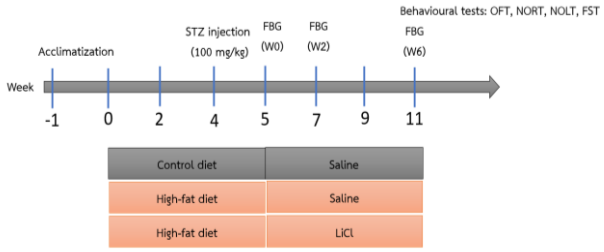

Figure 1. The experimental protocol of the study

2.2 Novel object recognition test (NORT)

The novel object recognition test (NORT) was appraised as described previously with some modifications (Janthakhin, Kingtong, & Juntapremjit, 2022; Juntapremjit & Janthakhin, 2021). Briefly, the test consisted of three phases: habituation phase, training phase, and test phase. During the habituation phase, mice were exposed to open-field box in the absence of objects for five minutes. Mice were then returned to their home cage. On the following day (training phase), mice were placed in the same open-field with two identical objects and were allowed to freely explore the environment and objects for 10 minutes. Twenty-four hours later (testing phase), mice were placed back in the open-field and at this time, allowed to explore a novel object and a familiar one for 10 minutes. During the experiment, the open-field box and objects were cleaned using 70% alcohol to eliminate the olfactory cue. Time spent exploring each object was collected in the test phase, counted when the mouse's nose was within 2 cm of the object. The percentage of time spent exploring the novel object versus the total object exploration time was determined. Data analysis of the behavioral tests was done manually from videotape recordings by the investigators, who were blinded to experimental groups.

2.3 Novel object location recognition test (NOLT)

The protocol carried out was an adapted version of that used by (Valladolid-Acebes *et al.*, 2013). The novel object location test was chosen as it has been demonstrated previously that it is a hippocampal-dependent task and it is non-aversive and less stressful (Barker, Bird, Alexander, & Warburton, 2007). Briefly, the assay was performed in the white plastic open-field box (40x40x25cm). The test consisted of two phases: (i) the training phase; mice were allowed to freely explore the box containing two identical objects (a glass bottle) for 10 minutes; and (ii) the retention phase, which was performed 3 hours after the training phase. Mice were allowed to re-explore the objects for 5 minutes where one object remained in the same position (a non-displaced object) but the location of another one was changed (a displaced object). The percentage of recognition index was calculated as the time spent exploring the displaced object divided by the total time spent exploring the displaced object and the non-displaced object X 100.

2.4 Open-field test (OFT)

The open-field test was used to measure anxiety-like behavior. Briefly, each mouse was placed in the center of the open square box (40x40x25cm) made from white and non-porous plastic, and the animals were allowed to freely explore the open-field box for five minutes. Time spent in the center of the open-field and the number of entries in the center of the open-field were recorded. Decreases in time spent in the center and number of entries in the center of the open-field indicate an increase in anxiety-like behavior of mice (Prut & Belzung, 2003).

2.5 Forced-swim test (FST)

The forced-swim test was used to evaluate depression-like behavior. The protocol used in this study was previously described with modifications (Bampi *et al.*, 2020). Mice were individually placed in a cylinder containing 20 cm of water maintained at 25°C. The immobility time was observed for 5 minutes. The immobility time was assigned when no additional activity was observed other than that required to keep the mouse's head above the water.

2.6 Measurement of blood glucose level

The blood glucose levels were evaluated one week after STZ injection (W0), two weeks after LiCl treatment (W2) and six weeks after LiCl treatment (W6). After a 6-hour fast, blood samples were collected from a small incision at the end of the mice's tail. A drop of blood was placed on a glucose strip, and blood glucose was measured with a glucose meter (Jiang *et al.*, 2021) according to the user instructions (ACCU-CHEK Guide, Roche, Thailand).

2.7 Data analysis

All data are expressed as means ± SEM and were analyzed using one-way ANOVA with Tukey's *post hoc* tests for multiple comparison; or two-way ANOVA with repeated measures when appropriate. Analyses were performed in GraphPad Prism version 5.0, and $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 The effect of lithium chloride treatment on blood glucose levels in diabetic mice

We first evaluated the effects of lithium chloride treatment on blood glucose levels in diabetic mice. Two way-ANOVA with repeated measures for the fasting blood glucose (FBG) levels revealed a significance of time effect ($F_{(2,52)} = 9.32$, $p < 0.001$), treatment effect, ($F_{(2,52)} = 37.31$, $p < 0.001$), and the interaction between time and treatment effect ($F_{(4,52)} = 4.84$, $p = 0.002$). *Post hoc* analysis revealed that after 2 weeks of treatment (W2), lithium chloride partially decreased the FBG level in diabetic mice ($t_{(17)} = 3.93$, $p < 0.001$) (Figure 2). However, the FBG level of the diabetic mice treated with lithium chloride was still higher than that of the normal control mice ($t_{(17)} = 4.76$, $p < 0.001$). Interestingly, after 6 weeks of treatment, lithium chloride effectively decreased the FBG level in diabetic mice ($t_{(17)} = 4.63$, $p < 0.001$) (Figure 2). Moreover, the FBG level of the diabetic mice treated with lithium chloride was not different from that of the normal control mice ($t_{(17)} = 2.25$, $p > 0.05$). These findings indicate that lithium chloride treatment was effective in decreasing the FBG levels in the diabetic mice.

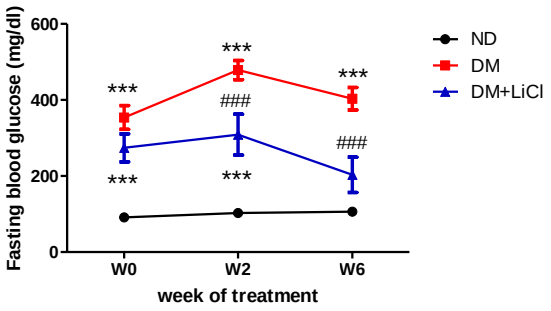

Figure 2. Lithium chloride treatment decreased the FBG levels in the diabetic mice. Normal control mice (ND) (n= 10), diabetic mice (DM) (n= 10), and diabetic mice treated with lithium chloride (DM+LiCl) (n= 9). ***$p < 0.001$ when compared to the normal control group (ND), $^{###}p < 0.001$ when compared to the diabetic group (DM).

3.2 The effect of lithium chloride treatment on cognitive functions in diabetic mice

The cognitive functions of these mice were evaluated in the novel object recognition test (NORT) and the novel object location recognition test (NOLT). We first evaluated the cognitive functions in NORT. An analysis of the percentage of recognition index by one sample *t* test revealed that the normal control mice showed a percentage of recognition index that differed from the chance level ($t_{(9)} = 2.30$, $p = 0.04$) (Figure 3A) indicating that the normal control mice (ND) could recognize the familiar object. However, the percentage of recognition index of the diabetic mice and the percentage of recognition index of the diabetic mice treated with lithium chloride did not significantly differ from the chance level ($t_{(8)} = 0.77$, $p = 0.46$ and $t_{(7)} = 0.62$, $p = 0.56$, respectively) (Figure 3A) indicating that the diabetic mice exhibited cognitive deficit in object recognition memory and the treatment with lithium chloride could not restore the cognitive abilities in object recognition memory of the diabetic mice.

We further evaluated the cognitive functions of these mice in the novel object location recognition test (NOLT), a task that is more hippocampal-dependent. An analysis of a percentage of recognition index by one sample *t*-test revealed that the normal control mice showed a percentage of recognition index that differed from the chance level ($t_{(9)} = 2.41$, $p = 0.04$). However, the percentage of recognition index of the diabetic mice did not significantly differ from the chance level ($t_{(9)} = 2.11$, $p = 0.06$). Interesting, the percentage of recognition index of the diabetic mice treated with lithium chloride significantly differed from the chance level ($t_{(8)} = 2.41$, $p = 0.04$). One-way ANOVA for the percentage of recognition index in NOLT revealed that the percentage of recognition index in NOLT differed among groups ($F_{(2,26)} = 7.15$, $p = 0.003$). *Tukey's post hoc* analysis showed that the percentage of recognition index of the

diabetic mice was lower when compared to the normal control mice ($p < 0.01$) (Figure 3B). Interestingly, the diabetic mice treated with lithium chloride showed an increase in the percentage of recognition index when compared to the diabetic mice (Figure 3B). These findings indicate that the diabetic mice exhibited cognitive deficit in object location recognition memory, and the treatment with lithium chloride could improve the cognitive performance in object location recognition memory of the diabetic mice.

3.3 The effect of lithium chloride treatment on affective functions in diabetic mice

We next evaluated the affective functions including anxiety-like behavior and depressive-like behavior in these mice. The anxiety-like behavior was appraised in the open-field test (OFT). One-way ANOVA for the time spent in the center showed that the time spent in the center differed among groups ($F_{(2,26)} = 3.81$, $p = 0.04$). *Tukey's post hoc* analysis showed that the diabetic mice exhibited a decrease in time spent in the center when compared to the normal control mice ($t_{(18)} = 2.57$, $p = 0.02$) (Figure 4A). The diabetic mice receiving lithium tended to show an increase in time spent in the center when compared to the diabetic mice, but without statistical significance ($t_{(17)} = 1.31$, $p = 0.21$). We also examined the number of entries in the center of the open-field and a one-way ANOVA revealed the difference in the number of entries in the center among groups ($F_{(2,26)} = 3.40$, $p = 0.04$). *Tukey's post hoc* test indicated that diabetic mice tended to exhibit a decrease in the number of entries in the center ($t_{(18)} = 1.89$, $p = 0.08$) while the treatment with lithium chloride increased the number of entries in the center of the diabetic mice ($t_{(17)} = 2.36$, $p = 0.03$) (Figure 4B). These results suggest that the treatment with lithium chloride could alleviate the anxiety-like behavior in diabetic mice.

We further evaluated the effect of lithium chloride on depressive-like behavior in a forced swim test (FST). One-way ANOVA for the immobility time revealed the difference among groups ($F_{(2,26)} = 11.50$, $p = 0.0003$). *Tukey's post hoc* test indicated that both diabetic mice and diabetic mice treated with lithium chloride exhibited increases in the immobility time when compared to the normal control mice ($t_{(18)} = 2.82$, $p = 0.01$ and $t_{(17)} = 7.34$, $p < 0.001$, respectively) (Figure 4C). Surprisingly, diabetic mice treated with lithium chloride exhibited increases in the immobility time when compared to the diabetic mice ($t_{(17)} = 2.14$, $p = 0.047$). These findings indicate that the diabetic mice exhibited depressive-like behavior and the treatment with lithium increased depressive-like behavior in the diabetic mice.

In the present study, we showed that the diabetic mice exhibited hyperglycemia, cognitive deficits in object recognition memory and object location recognition memory. They also demonstrated increases in anxiety-like behavior and depressive-like behavior. The treatment with lithium chloride at the dose of 100 mg/kg daily for 6 weeks could normalize the blood glucose level, improve cognitive function, and alleviate anxiety-like behavior in diabetic mice. However, the treatment with lithium chloride increased depressive-like

Figure 3. The effect of lithium chloride treatment on cognitive functions in diabetic mice. The diabetic mice exhibited cognitive deficit in novel object recognition test (NORT) (A) and cognitive deficit in novel object location recognition test (NOLT) (B). The treatment with lithium chloride could restore the cognitive function of the diabetic mice in NOLT. Normal control mice (ND) (n= 10), diabetic mice (DM) (n= 10), and diabetic mice treated with lithium chloride (DM+LiCl) (n= 9). $^{@}p < 0.05$ when compared to the chance level (50%), $^{**}p < 0.01$ when compared to the normal control group (ND), $^{\#\#}p < 0.01$ when compared to the diabetic group (DM).

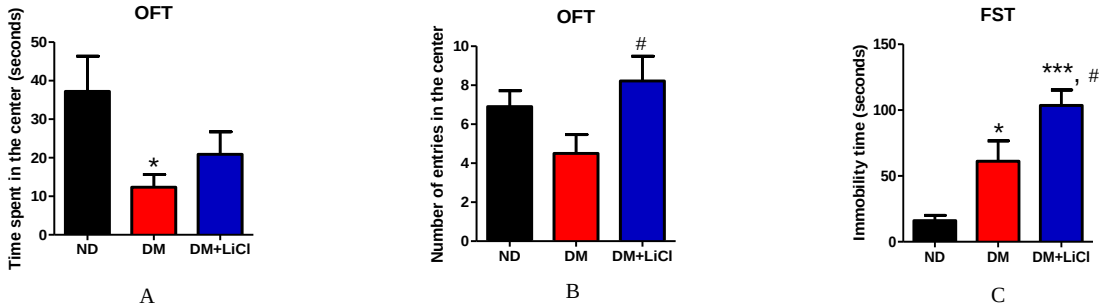

Figure 4. The effect of lithium chloride treatment on affective functions in diabetic mice. The time spent in the center of the open-field (A) and the number of entries in the center of the open-field (B) were quantified in the open-field test (OFT). The immobility time was quantified in the forced-swim test (FST) (C). Normal control mice (ND) (n= 10), diabetic mice (DM) (n= 10), and diabetic mice treated with lithium chloride (DM+LiCl) (n= 9). $^{*}p < 0.05$, $^{***}p < 0.001$ when compared to the normal control group (ND), $^{\#}p < 0.05$ when compared to the diabetic group (DM).

behavior in diabetic mice.

We first found that the administration of lithium chloride reduced fasting blood glucose in diabetic mice. This finding runs in parallel with the previous study demonstrating that lithium supplementation could reduce blood glucose levels in ob/ob mice, a genetic mouse model of diabetes (de Groot *et al.*, 2017). The mechanistic effect of lithium in regulating blood glucose levels may be due to its capacity to inhibit glycogen synthase kinase 3 (GSK3) which is a serine/threonine kinase and is an important component of PI3K/AKT insulin signaling. It has been demonstrated that overactivation of GSK3 is involved in the development of insulin resistance and type 2 diabetes (MacAulay & Woodgett, 2008; Wagman, Johnson, & Bussiere, 2004). So, lithium chloride exerts its anti-hyperglycemic effect possibly via inhibition of GSK3.

We further demonstrated that diabetic mice exhibited cognitive deficits in object recognition memory and object location recognition memory. Our results are consistent with previous studies reporting cognitive dysfunctions in diabetic animals (Cassano *et al.*, 2020; Janthakhin *et al.*, 2023). The treatment with lithium chloride restored the cognitive functions of the diabetic mice. These findings are in agreement with a previous study demonstrating the neuroprotective effect of lithium chloride in intracerebroventricular streptozotocin- treated rats (Ponce-Lopez *et al.*, 2011). However, the molecular mechanisms of how lithium chloride exerts the neuroprotective effects in diabetes-induced cognitive deficits remain unclear.

Lithium directly binds and inhibits GSK3, which has the two isoforms GSK3α and GSK3β. The phosphorylation on serine-21 and serine-9 inhibits the activity of GSK3α and GSK3β, respectively (Jope, 2003). Previous studies demonstrated that lithium chloride may play the neuroprotective role against cognitive impairments in several disease models including cognitive dysfunction induced by sevoflurane (SEV) anesthesia (Wang *et al.*, 2020), cognitive impairment in the repeated cerebral ischemia-perfusion mouse model (Xiao *et al.*, 2020), cognitive deficits in APP/PS1 mice, a genetic mouse model of Alzheimer's disease (AD) (Pan *et al.*, 2018), and cognitive dysfunctions in neuropsychiatric disorders (O'Leary & Nolan, 2015). Several mechanisms have been proposed to show how lithium chloride contributed to the improvement of cognitive functions, including the modulation of long-term potentiation (LTP) (Zhu *et al.*, 2007), enhancement of neurogenesis (G. Chen, Rajkowska, Du, Seraji-Bozorgzad, & Manji, 2000), decrease in neuroinflammation (Beurel & Jope, 2009) and reduction in apoptosis of neurons (Mines & Jope, 2011) and neurotrophic properties (Machado-Vieira, Manji, & Zarate, 2009) .

We next demonstrated that diabetic mice exhibited anxiety-like behavior, and the treatment with lithium chloride alleviated anxiety-like behavior in diabetic mice. Our results are consistent with the prior literature, both in clinical studies reporting that patients with diabetes mellitus showed anxiety symptoms more often than people without diabetes mellitus (Bickett & Tapp, 2016) and in streptozotocin-induced animal models of diabetes showing an increase in anxiety-like behaviors (Matinfar, Peeri, & Azarbayjani, 2021; Yuan, Zhang, Li, & Song, 2019). Interestingly, we demonstrated that lithium chloride administration alleviated anxiety-like behavior in diabetic mice. To the best of our knowledge, this is the first study to show that lithium chloride has a therapeutic effect on treating anxiety disorders in diabetic mouse model. Further study should investigate how lithium chloride could decrease anxiety-like behavior in diabetic mice at molecular and cellular levels.

We also found that the diabetic mice exhibited depressive-like behavior when evaluated in force-swim test (FST). This finding was in line with the previous results reporting depressive-like behavior in streptozotocin-induced diabetic mice (Hai-Na *et al.*, 2020; Zborowski *et al.*, 2020). However, in the present study, we found that the treatment with lithium chloride increased the level of depressive-like behavior in diabetic mice. This result is inconsistent with the previous studies that demonstrated the anti-depressive effect of lithium in an animal model of treatment-resistant depression (Kin *et al.*, 2019), in an animal model of depression induced by chronic unpredictable mild stress (CUMS) (Zhuo *et al.*, 2022) and in a rodent model of depression induced by combining repeated lipopolysaccharide pre-challenge followed by chronic mild stress (Ebeid *et al.*, 2021). It is important to note that although lithium has been widely used to treat the treatment-resistant depression (Del Matto *et al.*, 2020; Katz *et al.*, 2022) and has been the standard pharmacological treatment for bipolar disorder (Goodwin *et al.*, 2003), still the effectiveness of lithium chloride in the treatment of diabetes-associated depression remains unclear. So, more research is needed to evaluate the effect of lithium chloride treatment on diabetes induced-depressive-like behavior.

In the present study, the authors investigated the effects of lithium chloride administration in diabetic mice only by the intraperitoneal injection. This could be considered as a limitation. Thus, further study should investigate whether the different routes of administration of lithium (oral versus intraperitoneal administration) would influence the effects of the drug in decreasing the FBG levels, improving cognitive impairments, and alleviating anxiety-like behavior in diabetic mice.

4. Conclusions

In the present study, we showed that the diabetic mice exhibited hyperglycemia, and cognitive deficits in object recognition memory and object location recognition memory. The diabetic mice also demonstrated increases in anxiety-like behavior and depressive-like behavior. Interestingly, the treatment with lithium chloride at the dose of 100 mg/kg daily for 6 weeks could normalize the blood glucose level, improve cognitive function in object location recognition memory, and alleviate anxiety-like behavior in diabetic mice. Our findings provide added information about the therapeutic benefits of lithium against diabetes-associated cognitive and affective impairments. Further studies are needed to explore the mechanisms of how lithium chloride improves cognitive function and alleviates anxiety-like behavior in diabetic mice.

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