The Effect of Serotonin-Norepinephrine Reuptake Inhibitor Milnacipran on Anxiety-like Behaviors in Diabetic Mice

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Abstract

***Background**: Diabetes mellitus is a chronic disease that causes neuronal plasticity and increased hypothalamic pituitary adrenal (HPA) axis of stress disorders. The change in metabolism is reportedly associated with inadequate response to antianxiety and antidepressant agents. **Objective**: This study aimed to determine the effect of milnacipran antidepressants on anxiety-like behavior in mice with diabetes mellitus. **Methods**: Male ICR mice were divided into naive, stress, diabetes mellitus (DM), DM + stress groups to measure anxiety-like behavior. Diabetes mellitus was induced using alloxan, and electric footshock stress was used as a stressor for 14 consecutive days. Anxiety-like behavior was measured using the light-dark box (LDB) and elevated plus maze (EPM) test at days 0, 7 and 14. The antidepressant milnacipran (MIL) was given for 7 days, on days 8 to 14. On day 14, evaluation of anxiety-like behavior after administration of MIL was carried out in all groups using LDB and EPM tests. **Results**: The results showed that administration of milnacipran effectively ameliorated anxiety-like behavior in the non-DM, but not in the DM group, using the LDB test. A similar result was demonstrated in the EPM test showing the non-DM group's attenuation after milnacipran administration. **Conclusion**: The present results indicate that there is an inadequate attenuation of the anxiety-like behavior after treatment with milnacipran in diabetes conditions.*

***Keywords**: antidepressant, anxiety-like behaviors, diabetes mellitus, electric footshock stress, milnacipran*

Abstrak

Pendahuluan: Diabetes melitus merupakan penyakit kronis yang menyebabkan plastisitas saraf dan peningkatan aktivitas *hypothalamic pituitary adrenal (HPA) axis* yang berhubungan dengan gangguan psikologis terkait stres. Gangguan metabolik pada kondisi diabetes dilaporkan memperburuk respon individu dengan gangguan psikologis terkait stres terhadap obat anti-cemas dan antidepresan. **Tujuan**: Penelitian ini bertujuan untuk mengetahui pengaruh pemberian antidepresan milnacipran terhadap perilaku kecemasan mencit diabetes melitus. **Metode**: Mencit ICR jantan dibagi menjadi kelompok naif, stres, diabetes melitus (DM), DM + stres, untuk mengukur *anxiety-like behavior*. Diabetes melitus diinduksi menggunakan aloksan, dan *electric footshock stress* digunakan sebagai stressor selama 14 hari berturut-turut. *Anxiety-like behavior* diukur menggunakan uji *light-dark box (LDB)* dan *elevated plus maze (EPM)* pada hari ke 0, 7, dan 14. Antidepresan milnacipran (MIL) diberikan selama 7 hari, pada hari ke-8 sampai 14. Pada hari ke-14, evaluasi *anxiety-like behavior* setelah pemberian MIL dilakukan pada kelompok naif, stress, stress + MIL, DM + stress, DM + stress + MIL menggunakan uji LDB dan EPM. **Hasil**: Pemberian milnacipran hanya efektif memperbaiki *anxiety-like behavior* pada kelompok non-DM (stres + MIL), tetapi tidak pada kelompok DM dengan uji LDB. Uji EPM menunjukkan hal yang sama, kelompok DM tidak menunjukkan perbaikan *anxiety-like behavior* setelah pemberian milnacipran. **Kesimpulan**: Pemberian antidepresan milnacipran tidak memperbaiki *anxiety-like behavior* pada mencit diabetes mellitus.

Kata kunci: antidepresan, *anxiety-like behavior*, diabetes melitus, *electric footshock stress*, milnacipran

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INTRODUCTION

Diabetes mellitus is a metabolic disease that decreases the quality and life expectancy (Qiu *et al*., 2016). The increasing prevalence of diabetes causes socioeconomic and psychological pressures, a big challenge for individuals (Li *et al*., 2019). Structural and neurophysiological changes in the central nervous system caused by diabetes is associated with cognitive deficits and psychiatric disorders (Myers *et al*., 2013; Qiu *et al*., 2016). In diabetes mellitus, there is a decrease in hippocampal neurogenesis and neuroplasticity changes associated with depression and anxiety disorders (Ho *et al*., 2013).

Depression is a heterogeneous disorder due to changes in monoamine neurotransmitters in the brain, especially norepinephrine (NE) and serotonin (5-HT) (Li *et al*., 2020). Depression as comorbid diabetes was classically introduced 300 years ago (Moulton *et al*., 2015). The likelihood of depression in diabetes mellitus patients ranges from 10 - 15%, two times greater than in the non-diabetic population (Sartorius, 2018). Comorbid depression in diabetes mellitus patients is also associated with poor outcomes. Diabetes mellitus patients with depression have more difficulty controlling blood glucose levels and have unhealthy lifestyles (Li *et al*., 2019). In vivo studies show that depression is determined by measuring experimental animals anxiety levels (Kamei *et al*., 2003).

Improvement of mental conditions, glycemic control and good outcomes is commonly obtained through pharmacological therapy, psychotherapy or a combination of both (Li *et al*., 2019). Various types of drugs that act on the central nervous system have been used clinically as therapeutic agents. However, an effective treatment strategy for anxiety and depression in diabetes mellitus has not been established.

The effectiveness of serotonin noradrenaline reuptake inhibitors (SNRI) on anxiety disorders has been established in clinical trials. However, the therapeutic effect of these drugs varies depending on the type of anxiety disorder (Miyamoto *et al*., 2004). Milnacipran is an SNRI group that simultaneously inhibits 5-HT and NE reuptake (Bourin *et al*., 2005). Murthi & Vaillancourt (2019), in their research, proved that 5-HT might also act as a factor in maintaining normoglycemia. The use of SNRI in the animal study of anxiety-like behaviors showed an increase in the frequency and time of mice entering the open arms (Takeuchi *et al*., 2010). In addition, milnacipran reduces immobility time in stress-induced mice *(Mochizuki et al*., 2002). However, there is a lack of evidence showing the efficacy of milnacipran on the anxiety-like behavior of diabetic mice. Thus, the present study investigated the effect of milnacipran on anxiety-like behaviors in diabetes mellitus.

This study measured anxiety-like behavior after milnacipran treatment in diabetes mellitus mice using the light-dark box (LDB) and elevated plus maze (EPM) tests. These methods are based on the principle that rodents do not prefer to stay in open spaces and heights (Bisong *et al*., 2018). The diabetes mellitus model was developed using alloxan. Alloxan induces diabetes through a partial degradation mechanism of pancreatic beta cells, which affects the quality and quantity of insulin (Ighodaro *et al*., 2017). The electric footshock as a stress stimulus was used to induce anxiety-like behaviors in mice. It is known that many studies effectively produce an adequate anxiety-like state and fear response through inescapable electric foot shock exposure (Silva *et al*., 2020).

MATERIALS AND METHODS

Materials

Milnacipran hydrochloride (Ace Pharmaceuticals, Japan), alloxan monohydrate (Sigma-Aldrich, Germany), citrate buffer pH 4.5 (Sigma-Aldrich).

Tools

Light dark box test apparatus, elevated plus maze test apparatus, electric footshock apparatus, stopwatch, Easy Touch ® blood glucose monitoring system.

Animals

Male (6 - 10 weeks old) ICR mice, weighing between 26 and 30 g, were used. All mice were maintained at a regulated temperature (25°C ± 2°C) and humidity (60 ± 10%) in a 12:12 h diffuse light/dark cycle with free access to food and water. All experiments were performed at the Animal Research Laboratory of the Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia. The design and conduct of the study were in accordance with Helsinki's guidance on animal welfare. All effort was done to reduce the animal number and suffering.

Experimental design and treatments

Mice were divided into naive, stress, diabetes mellitus (DM), and DM + stress groups in experiment I (n = 8). Diabetes mellitus was induced using a single injection of alloxan. An Anxiety-like state was induced by electric footshock stimulus (0.45 mA, 1 s) 30 times with 9-second intervals for 14 consecutive days. Naive mice as the control group received a normal saline

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injection and were placed in the electric chamber for 6 minutes a day without shock stimulus. Evaluation of anxiety-like behavior was measured using the light-dark box (LDB) and elevated plus maze (EPM) test at days 0, 7 and 14. In experiment II (n = 12), an evaluation of anxiety-like behavior after administering milnacipran (MIL) was carried out in the naive, stress, stress + MIL, DM + stress, DM + stress + MIL on day 14. The antidepressant milnacipran at a dose of 20 mg/Kg, reportedly effective in ameliorating anxiety-like behavior (Takeuchi *et al.*, 2010) was given for seven days, on days 8 to 14.

Milnacipran was dissolved in saline and administered orally. The drug was given once a day.

Alloxan-induced diabetes mellitus

Mice were induced with diabetes mellitus by injecting alloxan 170 mg/Kg intraperitoneally (Ighodaro *et al.*, 2017). Alloxan monohydrate was dissolved in a citrate buffer pH 4.5. The blood glucose levels of the mice were checked 48 h after injection with a strip test. Mice with blood glucose levels ≥ 200 mg/dL or 11.1 mmol/L are considered to have diabetes mellitus.

The electric footshock stress procedure

Mice were put into a box measuring 18 x 15 cm with a grid floor from steel,the middle of the box was given a bulkhead. Stress was induced by an inescapable electric footshock with a voltage of 0.45 mA, 60 volts, as previously described (Seo, 2018). A set of electric footshocks has a duration of 1 sec, repeated 30 times at 9-sec intervals. The stress-induced group received two sets of electric footshocks per day. Electric footshocks were exposed for 14 days.

Light dark box (LDB) test

The protocol was performed following the previous study (Zhang *et al.*, 2020). The instrument used consists of two boxes separated by a bulkhead and connected by a door (5 x 5 cm). The light box (27 x 18 x 18 cm) is lit with a white 60-watt incandescent bulb. The dark box (18 x 18 x 18 cm) is completely black. Both are equipped with cameras to record the movements of mice. Mice were placed in a light box facing the door and allowed to explore both boxes for 5 min. The time spent in each box was recorded. The percentage of time spent in the light box was also calculated.

Elevated plus maze (EPM) test

The protocol was performed following the previous study (Walia *et al.*, 2019; Zhang *et al.*, 2020). The apparatus consists of two open and closed arms (each 30 × 6 cm). Closed arm surrounded by 15 cm wall. The arms cover the central area of the maze with a size of 5 × 5 cm. The apparatus is placed 50 cm above the floor. The mice were placed in the centre area, facing the open arms and exploring the arms for 5 minutes. A camera was positioned above the apparatus to record mice activity. The number of entries to the open arm was recorded, and the percentage was calculated. The movement of mice was calculated when all four legs entered the arm.

Statistical analysis

Data are presented as mean ± SEM. Measurement of anxiety-like behavior using the LDB and EPM tests at several time points were analyzed using the two-way ANOVA test, followed by the Bonferroni post-hoc test. Meanwhile, the measurement of anxiety-like behavior after milnacipran administration was analyzed using the one-way ANOVA test, followed by the Bonferroni post hoc test. The difference was considered significant if $p < 0.05$ (95%).

RESULTS AND DISCUSSION

The effect of stress induction on anxiety-like behaviors

This study observed stress-induced diabetes mellitus mice using the electric footshock method with the light-dark box (LDB) and elevated plus maze (EPM) tests. The LDB test showed that the stress group significantly reduced time spent in the light box compared to the naive group, seen on days 7 and 14. In addition, the DM + stress group also showed the same results compared to the DM group (Figure 1).

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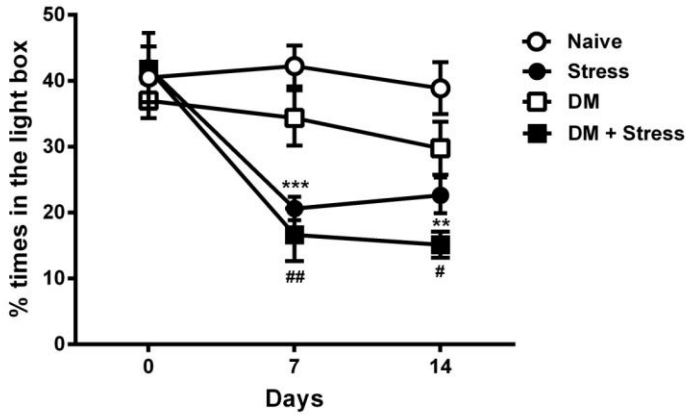

Figure 1. The effect of electric footshock stress induction on anxiety-like behaviors in mice with diabetes mellitus measured by light-dark box test (mean ± SEM) of 8 mice. **p < 0.01, ***p < 0.001 vs naive group. $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 vs DM group. DM, diabetes mellitus

On the other hand, EPM test results showed no difference in the percentage of open-arm exploration between the stress and naive groups. However, a significant difference was found in the DM + stress group, which had a lower percentage of open-arm exploration than the inexperienced group at day 14 (Figure 2).

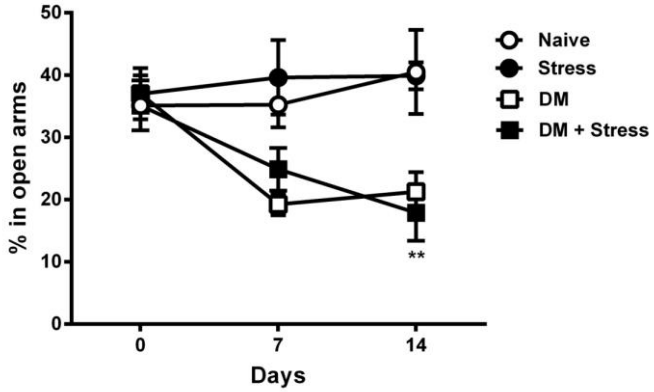

Figure 2. The effect of electric footshock stress induction on anxiety-like behaviors in mice with diabetes mellitus measured by elevated plus-maze test (mean ± SEM) of 8 mice. **p < 0.01 vs naive group. DM, diabetes mellitus

This result suggests that the present method of stress induction successfully induces anxiety-like behavior reflected in LDB, but not the EPM test. This condition might be due to the severity of stress stimulus during installation that may produce a differential anxiety-like response to specific situations, such as light space or open space stimulus. Moreover, it is known that anxiety with complex emotional states may not be generalized as an expression of a single established behavior.

The difference in EPM conditions and equipment was a factor in the different results. Previous studies of stress induction by the electric footshock method in experimental animals 24 and 48 h before measurement showed no difference in the percentage of time spent in mice in open arms (Grahn *et al*., 1995). Another study comparing the effectiveness of test results between elevated zero maze (EZM) and EPM during repeated trials showed behavior in EZM remained relatively stable for several trials and was more suitable than EPM for anxiety experiments (Tucker & McCabe, 2017).

However, the results of the two tests in this study indicate that the condition of diabetes mellitus with stress possibly increases anxiety-like behaviors in experimental animals compared to diabetes mellitus and/or naive conditions alone. This result is in accordance with previous studies that showed an increase in the severity of anxiety in streptozotocin-induced diabetes mellitus mice (Yuan *et al*., 2019).

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The effect of milnacipran treatment against anxiety-like behaviors

The results showed that administration of milnacipran effectively ameliorated anxiety-like behavior measured with the LDB test in the non-DM group (stress + MIL), but not in the DM group (DM + stress + MIL, Figure 3). This result was demonstrated by the increased time spent in the light box only in the stressed group given milnacipran.

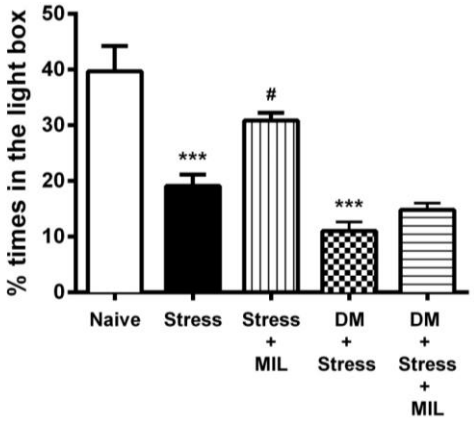

Figure 3. The effect of milnacipran on percentage time spent in the light box in diabetes mellitus mice with anxiety-like behaviors, measured by light-dark box test at day 14 (mean ± SEM) of 12 mice. ***p < 0.001 vs naive group. #p < 0.05 vs stress group. DM, diabetes mellitus; MIL, milnacipran

Similarly, the EPM test results showed that milnacipran did not affect the anxiety-like behavior in the stressed DM group. The present study showed no attenuation in the decreased percentage of open-arm exploration compared to the group without milnacipran administration (Figure 4).

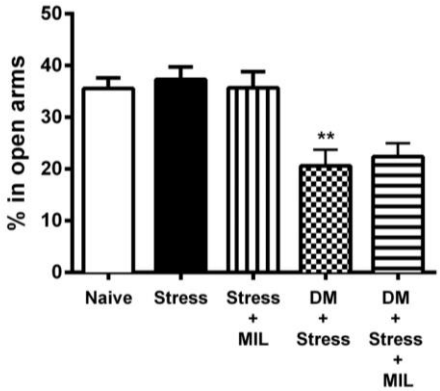

Figure 4. The effect of milnacipran on the percentage of open-arm exploration in diabetes mellitus mice with anxiety-like behaviors was measured by elevated plus maze test at day 14 (mean ± SEM) of 12 mice. **p < 0.01 vs naive group. DM, diabetes mellitus; MIL, milnacipran

Diabetes mellitus has been reported to increase anxiety-like behaviors. Animals with anxious behavior exhibit lower extracellular serotonin levels than normal animals. It is said that decreased serotonin levels is closely associated with anxiety. Furthermore, it is known that there is a change in serotonin activity in diabetic conditions. The animal model for DM exposed to stress stimulus demonstrates lower extracellular serotonin levels in the hypothalamus than those without stress induction and non-diabetic stressed animal (Thorré *et al*., 1997). In addition, serotonin modulates noradrenaline (NA) activity. A decrease in serotonin levels is associated with reducing NA levels (Moret et al., 2011). Milnacipran, an antianxiety drug used in the present study, is an SNRI drug that inhibits serotonin and NA uptake with the same potency, without any affinity for dopaminergic transporters (Bourin *et al*., 2005; Li *et al*., 2020).

The present results show that milnacipran is not effective at attenuating anxiety-like behaviors in diabetes mellitus. The effect of milnacipran may be lowered due to the changes in the brain's serotonin and NA systems. However, it is also possible that increasing the dose of milnacipran may attenuate anxiety-like

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behaviors in diabetes mellitus. The study by Takeuchi *et al.* (2010) showed a dose-dependent of milnacipran increased the time spent in open-arms and the number of open-arm entries. Further research is needed to clarify this issue and the exact mechanisms.

Moreover, previous research has shown a correlation between changes in the brain's glial cells of diabetic mice with anxiety phenotypes. In addition, it was reported that astrocyte activation is found in the hippocampus of diabetic mice, the area that contributes to the development of fear memory and depression (Saravia *et al*., 2002). Studies showed that the marker protein for astrocyte activation, GFAP, is precisely regulated together with the upregulation of IL-6, indicating a neurological inflammatory response in the CNS of diabetic mice (Qiao *et al*., 2016; Yuan *et al.*, 2019). This condition suggests that anxiety states in diabetes mellitus comprisecomplex changes in the neurotransmission system that may affect the efficacy of pharmacological treatment. It remains to be explored whether the different classes of antianxiety demonstrate distinctive tolerability for treating anxiety in diabetic conditions.

CONCLUSION

It is concluded that the antianxiety effect of milnacipran has deteriorated in diabetes mellitus mice. Furthermore, it is suggested that there is a differential anxiety-like response from the EPM and LDB method for anxiety measurement.

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