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The effects of donepezil on anxiety- and depression-like behaviors in diabetic rats and the role of nitric oxide modulators

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ABSTRACT

Aims: The aim of the present study was to evaluate the effect of an acetylcholinesterase inhibitor donepezil on the diabetes-induced anxiety and depression and the role of nitric oxide in these effects.

Methods: Thirty male Wistar rats were randomly divided into 5 groups (6 rats each): (I) normal control group, (II) untreated diabetic group, and Groups (III-V) diabetic rats received donepezil at a dose of 4 mg/kg orally for twenty days after the first 30 days of diabetes. Group 4 also received 20 mg/kg i.p., L-NAME simultaneously with donepezil for the last 20 days, while group 5 received 40 mg/kg i.p., L-Arginine during this period. A single dose of streptozotocin was used to induce experimental type 1 diabetes.

Results: Anxiety-like behaviors were assessed using the open field test (OFT), and depression-like behaviors were estimated using the forced swim test (FST). In the OFT, all diabetic rats spent less time in the center and engaged in less exploratory behavior than the control group. The number of lines crossed where locomotor activity was assessed did not differ significantly between groups. In the FST, duration of immobility increased significantly in diabetic groups compared to the control. Donepezil administration did not affect either depression or anxiety responses. Moreover, donepezil plus L-arginine increased diabetes-induced depression significantly.

Conclusion: These findings may suggest that cholinergic and nitrergic systems may interact on depression-like behaviors in diabetic rats.

Keywords: Diabetes, depression, anxiety, acetylcholinesterase, nitric oxide

INTRODUCTION

Diabetes mellitus (DM) is one of the main global public health problems that is becoming more serious over time, and it is frequently associated with depression, which leads to a lower quality of life and a worse long-term prognosis.[1] Essentially, as seen in major chronic diseases, there is a reciprocal relationship between diabetes and depression, such that those with diabetes are more likely to develop depression, while those with depression are more likely to develop diabetes.[2] Because the consequences of each condition are aggravated by the presence of the other, a vicious circle develops spontaneously and has a negative impact on disease pathogenesis. Nonetheless, this bidirectional relationship is not entirely clear, and the pathophysiologic mechanisms underlying between these two diseases remain a mystery. Hyperglycemia is independently associated with depression.[1] However, the exact mechanism of anxiety and depression development under the influence of hyperglycemia has not been fully characterized. Understanding the etiology of diabetes and identifying the points of intersection of its close relationship with depression will thus be critical for the development of treatment strategies.

Cholinergic transmission is known to play a crucial role in cognitive functions containing learning and memory.[3-6] In line with this perspective, changes in acetylcholine (ACh) levels are involved in the regulation of learning and memory or depression-like behaviors, it is known that brain ACh levels are elevated in depressed patients and remain at this level as long as depression persists,[7] and acetylcholinesterase (AChE) activity is the main regulator of ACh levels.[8] However, there are inconsistent results regarding the effect of AChE activity on these functions in cognitive dysfunctions. While decreased AChE activity, which results in increased ACh levels, is associated with impaired cognitive function,[4] others have shown that increased AChE activity similarly leads to impaired cognitive function.[5,6] A similar contradiction is seen in depression-like behavior in experimental animals after

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pharmacologically reducing ACh release or increasing its expression. In a study using physostigmine, an AChE inhibitor, depression-like behaviors were observed to increase,[3] while there is also a report showing that increasing the amount of acetylcholine through ACh inhibition exhibits antidepressant properties.[9] Nitric oxide (NO), another neurotransmitter known to have an effect on depression and anxiety, is defined as a durable gaseous free radical and is also referred to as an crucial biomodulator in the body.[10] The neuromodulatory role of NO in the central nervous system and its possible role in central nervous system-related disorders have been observed in experimental studies, where L-arginine, an NO precursor, shows an anti-anxiogenic (anxiolytic) effect due to restraint stress in rats. A similar effect was observed at low dose (10 mg/kg) of L-NAME, a nitric oxide synthase (NOS) inhibitor, whereas high dose L-NAME (50 mg/kg) further worsened the existing behavioral impairment.[10] Furthermore, NOS inhibitors, L-NAME,[11,12] 7-nitroindazole (7-NI),[11] and aminoguanidine,[13] are known to improve stress-induced anxiety and depression in different stress models causing anxiety and depression, and this improvement is dose-dependent,[11,13] and these inhibitors achieve this improvement by different mechanisms,[11] L-NAME modulates plasma NO and corticosterone, while 7-NI modulates brain NO stimulation. As it can be understood from the reports, the dose-dependent change in the responses and sometimes paradoxical results at similar doses; in addition, the biphasic properties of these substances increase the confusion of the researchers on the subject and reveal the importance of new studies in determining the mechanism of action of NO on depression and anxiety. This study was designed to investigate the effect of donepezil, an AChE inhibitor, on depression- and anxiety-like behaviors in STZ-induced diabetic rats and also the role of NO modulators; precursor (L-arginine) and inhibitor (L-NAME) in these effects

METHODS

Experimental Design

This study was carried out at Necmettin Erbakan University Experimental Medicine Research and Application Center after obtaining ethics committee approval (2022/015). Wistar albino rats, 12-15 weeks of age, with an average weight of 200-250 g were used in the study. All rats were housed in normal rat cages, four per cage, in a controlled environment at 22±2 °C, 50% humidity and quiet conditions, with ad libitum access to water and food, under 12/12 h light-dark cycles.

Formation of Groups

Our study was completed using 30 rats (n=6) in 5 groups, and the rats were randomly assigned to the groups. Diabetes was induced experimentally in all groups except the control group (CON). Diabetes groups were seperated into four subgroups as diabetic control group (DIA), donepezil group (DON), donepezil plus L-NAME (L-NAME) group and donepezil plus L-arginine (L-arg) group. Injections were administered by the same investigator at the same time of the day, and an equal volume of vehicle was either injected or gavaged into the rats that received no supplementation. At the end of the study, all rats were subjected to behavioral tests. The experimental protocol in the present study is schematically represented in **Figure 1**.

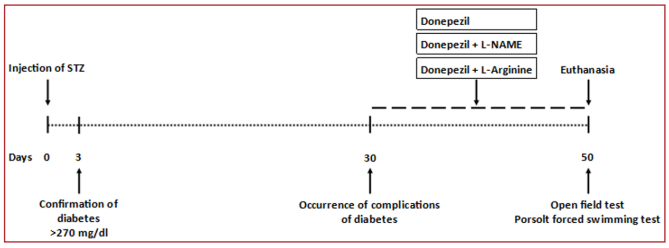

Figure 1. Schematic presentation of the protocol used for the study

Induction of Diabetes

STZ was used to induce experimental diabetes. All rats in the diabetes groups were administered 55 mg/kg dose of STZ intraperitoneally as a single dose after 12-hour fasting.[14] STZ was dissolved in citrate buffer (10 mM, pH 4.5), the control group was injected with the same volume of citrate buffer only.[14] Blood was taken from the tail vein 72 hours after the injections and blood glucose levels were measured. Rats with blood glucose levels above 270 mg/dl were considered diabetic and included in the study.

Drugs and Administration

After STZ induced diabetes was confirmed, we waited for 30 days to occur the complications of diabetes. Subsequently, donepezil at a dose of 4 mg/kg was administered orally by gavage to the DON, L-NAME and L-arg groups at the same time every day.[15] In another group, L-NAME was administered intraperitoneally 30 minutes after the administration of donepezil in a volume of 20 mg/kg.[16] The rats in the L-arg group were administered L-arginine 40 mg/kg intraperitoneally at the same time as the rats in the L-NAME group.[17] Donepezil, L-NAME and L-arginine were administered for a total of 20 days and behavioral tests were performed on the last day of administration. All drugs were obtained from Sigma-Aldrich Chemical Co.

Behavioral Tests

Behavioral tests were performed in a separate room that was never used during any phase of the study. The room did not contain any noise or odor to distract the animals. There was at least a 2-hour interval between behavioral tests and all rats were acclimated to the room environment before the tests began. Behavioral tests were achieved and videotaped by the alike person. The recordings were evaluated blindly by the other investigators.

Open Field Test

In our study, we applied an open field test to assess anxiety-like behaviors. Each rat was gently placed in the middle of a well-lit open field arena (80 cm×80 cm, height 40 cm, made of black acrylic material) and permitted to explore the arena freely for 5 minutes. The open field arena was separated into 16 equal squares, and the 4 sections in the center and 12 sections on the edge were named center and periphery, respectively. The time spent in the center was recorded by calculating the time spent in the 4 squares located in the middle zone. Rearing number (vertical movement) was scored as 1 point if the rats stood on their hind legs and crossing number (horizontal movement) was scored as 1 point if at least 3 paws entered the same square.[18]

The arena was cleaned with ethanol after each trial to ensure that the residual odor did not affect the next rat

Forced Swim Test

Rats were placed in an acrylic cylinder (24 cm diameter and 60 cm height) filled with 25°C water and allowed to swim freely. The depth of the water was such that their tails did not touch the ground, they could not get out, and they could swim freely (40 cm). Rats were considered immobile when they swam in the water without struggling and keeping their heads above water. They were subjected to a 15 min acclimatization test the day before the test, followed by another 5 min of forced swimming on the day of the test. The first and last 1 min were subtracted and the immobility time in seconds was recorded for the intervening 3 min.[18] The rats were dried with a towel and left to dry completely in a heat source for 20 min after each swimming session. The cylinders were prepared for a new test by refilling with water at the same temperature after each session.

Statistical Analysis

All results were expressed as mean value±standard deviation (SD). Statistical differences were determined by analysis of variance (ANOVA, SPSS 20.0) followed by Tukey post-hoc analysis. The statistical significance level was determined as $p<0.05$.

RESULTS

Forced Swimming Test

The forced swim test was used to assess depression-like behavior by measuring the immobility time of each rat (**Figure 2**). Duration of immobility increased significantly in all diabetic groups compared to the control group (p=0.000 vs all other groups). According to these results, our study shows that STZ-induced diabetes causes depression-like behaviors in rats. Rats in the L-NAME treated group showed similar results as in the DIA and DON groups. But, in donepezil administered diabetic rats, L-arginine increased diabetes-induced depression-like behaviors significantly (p=0.000 vs CON; p=0.001 vs DIA; p=0.014 vs DON; p=0.004 vs LNAME).

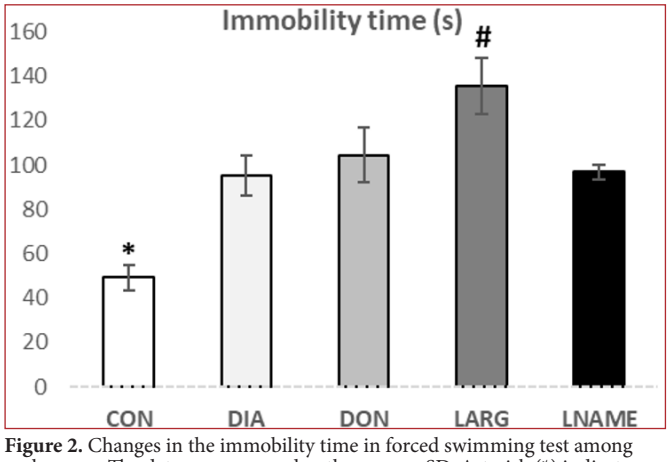

Figure 2. Changes in the immobility time in forced swimming test among each group. The data are expressed as the means±SD. Asterisk (*) indicates significance compared with other groups. Hash (#) indicates significance compared with other diabetes groups. p<0.01; one-way ANOVA.

Open Field Test

The open field test was used to assess anxiety-like behavioral responses in a novel environment. The time spent in the center was found to be lower in diabetic rats compared to the control group (p=0.003 vs DIA; p=0.005 vs DON and LARG; p=0.006 vs LNAME; **Figure 3A**). There was no significant difference between all diabetic groups in terms of time spent in the center (p>0.05, **Figure 3A**). In addition, the number of rearings, which we used as an indicator of exploratory behavior, was also higher in the CON group than in the diabetic groups (p=0.000 vs DIA, LARG and LNAME; p=0.001 vs DON; **Figure 3B**). The movement of the rats in the horizontal plane (crossing count) was used to evaluate locomotor activity. This activity was higher in the CON group compared to the diabetes groups, but this trend did not reach statistical significance (**Figure 3C**). It was found that donepezil, L-NAME or L-arginine administration to diabetic rats did not cause a significant difference in terms of both vertical movements and movements in the horizontal plane (p>0.05).

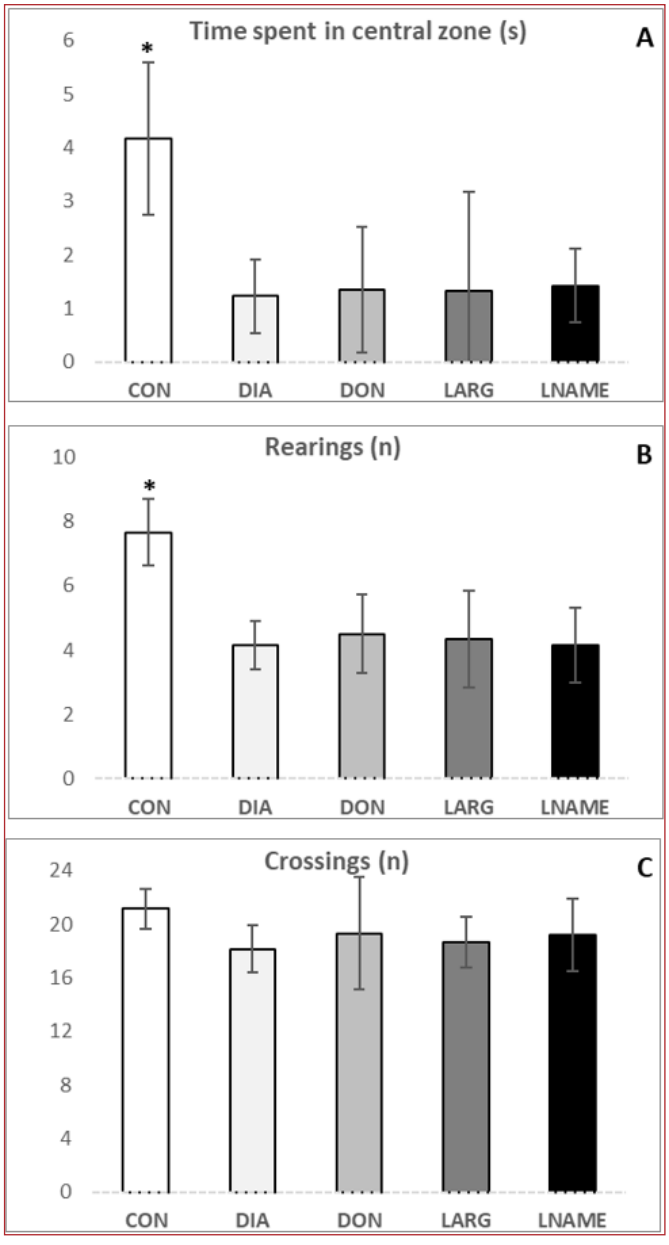

Figure 1. Effect of donepezil, L-NAME and L-Arginine on (A) time spent in central zone (B) number of rearing (C) number of crossing in the open field test in stz-induced diabetic rats. The data are expressed as the means±SD. Asterisk (*) indicates significance compared with other groups. p<0.01; one-way ANOVA.

DISCUSSION

The current study used a well-established method for creating diabetes in order to examine the effect of donepezil, an acetylcholinesterase inhibitor on diabetes-induced anxiety and depression and the role of NO.

To the best of our knowledge, our study is the first to demonstrate the effect of donepezil on depression and anxiety-like behaviors in STZ-induced diabetic rats and the role of NO in this effect. In this study, diabetes-induced reduction both in time spent in the center and exploratory activity in the OFT and increase in duration of immobility in the FST. Donepezil had no effect on either anxiety- or depression-like behavior in diabetic rats. However, L-arginine, administered rats, there was an increase in the duration of immobility in the FST associated with increased levels of depression.

Injection of STZ in rats leads to persistent hyperglycemia within approximately 48 hours and, in fact, hyperglycemia is responsible for diabetes-related complications, including cognitive and behavioral ones.[1] In fact, the pathophysiology of these complications is multifactorial and may be interrelated in some way and this complex relationship may often turn into a vicious circle. In our study, blood glucose levels were measured from the tail vein 72 hours after STZ injection and those above 270 mg/dl were considered diabetic. During the study period, blood glucose levels were above 270 mg/dl in all diabetic groups and there was no statistically significant difference between the all-diabetic groups (DON, L-NAME and L-ARG) in weekly measurements, so the relevant data are not presented here. Diabetes was accompanied by loss of weight in all groups (data not given). There was no significant difference in body weight between all diabetic groups and this was the same until the end of the study.

In our study, immobility time, which is considered as an indicator of depression in the forced swim test, increased in the diabetic groups compared to the control group (**Figure 2**). In the open field test, the time spent in the center and the number of rearing, which we evaluated as exploratory behavior, were lower in the diabetic groups (**Figure 3A-B**), indicating that anxiety was induced in the diabetic groups. Neurobiological studies suggest different mechanisms for the occurrence of depression-like behaviors in diabetic rats. Diabetes causes increased hippocampal levels of proinflammatory cytokines, such as TNF-α, NF-κB and IL-6, decreased neurotrophic factors, such as BDNF, NGF and IGF-1,[19] increased hippocampal lipid peroxidation and plasma glycated hemoglobin levels, and decreased antioxidant status as assessed by GSH,[20] and decreased insulin receptor phosphorylation in rat hippocampus, decreased ATP and glucose transporter 4 (GLUT4) expression,[21] decreased levels of glutamate, serotonin and dopamine in the hippocampus and associated hippocampal neuron apoptosis,[22] impaired regulation of hippocampal oxidative enzymes (GSH, SOD, CAT, LOOH),[23] increased brain proinflammatory cytokines (TNF-α, IL-6 and IL-1β) and MDA levels, and reduced SOD; CAT and GSH activity.[24] One of the important neurotransmitters in the pathophysiology of diabetic depression is ACh, and patients suffering from depression have high brain ACh levels and remain at this level as long as depression persists.[7] Donepezil is a synthetic AChE inhibitor that enhances cholinergic function by increasing the amount of ACh in the central nervous system and thus its interaction with the relevant receptors. Pharmacologic inhibition of AChE improves serum IL-6, brain noradrenaline and serotonin levels in diabetic rats, and donepezil is suggested to act through these mediators in the recovery of diabetes-induced brain oxidative damage.[25] However, donepezil was not effective in the regulation of proinflammatory cytokines such as hippocampal TNF-α and IL-1β and antioxidant enzymes such as GSH-SOD in type 2 diabetic rats.[26] Moreover, according to the available evidence, pharmacologic inhibition of AChE showed anxiogenic[3] and anxiolytic[9] effects, indicating that the subject is still in need of clarification and new studies are needed in this field. In this study, we do not know the effect of donepezil on these parameters (proinflammatory cytokines, oxidative parameters, etc.), which is the first limitation of our study. Nevertheless, we know that the effect of donepezil on diabetes-induced depression-like behaviors was not statistically significant in our study ($p>0.05$, **Figure 2**). These differences in the results may be attributed to the differences in the dose and duration of administration of the drug, the differences in the age, race and perhaps sex of the animals used, and the differences in the experimental stress models; a recent study reported that low doses of donepezil showed antidepressant effects but high doses had no effect,[27] which indicates that our results are not surprising.

Increased experimental evidences suggests that NO is associated with mood disorders such as anxiety and depression, which affect large numbers of people globally and have increasing morbidity. Epithelial and neuronal-derived NOS have been shown to be expressed in brain regions responsible for depression-anxiety responses and memory formation, such as the hypothalamus, hippocampus and amygdala.[28-30] Stress-inducing conditions lead to an increase in hippocampal NO levels in experimental animals, and NO induces the production of proinflammatory cytokines via microglia and these inflammatory mediators are involved in the occurrence of neuronal damage leading to the emergence of deviated behavioral symptoms associated with anxiety and depression. The reduction of NO levels as a result of L-NAME administration, which is responsible for the prevention of damage by modulating the production of inflammatory mediators,[31] indicates the magnitude of the effect of NO in the physiopathology of depression. NO not only induces depressive behaviors by inducing the production of proinflammatory cytokines, but also leads to impairments in oxidative stress parameters.[13] Essentially, proinflammatory cytokine production and this condition interact with each other in a vicious circle. Other studies demonstrating the effect of systemic NO inhibition on stress-induced anxiogenic behaviors suggest that L-NAME,[11] and L-NAME and 7-NI[12] cause anxiolytic effects. In our study, rats receiving L-arginine showed high immobility time in the forced swim test than the other diabetes groups, which means that the increased amount of nitric oxide by L-arginine administration worsens the depression-like behavior and is consistent with the studies mentioned above. In our study, diabetic rats receiving L-arginine were also receiving donepezil and we cannot say whether this effect is due to L-arginine alone or its interaction with donepezil, which is another limitation of our study. Nonetheless, NO modulates ACh release, especially exogenous NO is more effective in the upward induction of ACh release than endogenous NO.[32] Our speculative, but at the same time, need-to-be-proven explanation for the fact that the group receiving L-arginine exhibited more severe depressive behaviors than the other diabetes groups is as follows: Increasing the amount of NO by systemically administering L-arginine leads to an increase in ACh release, and since ACh and NO individually induce depressive behaviors, this result observed in the L-ARG group is a result of a cumulative effect due to **a)** the increase in NO by administering L-arginine and the stimulation of ACh release by NO **b)** and the increase in the amount of ACh by decreasing

AChE enzyme activity due to donepezil administration. On the other hand, there are also experimental evidences that points in the opposite direction. In contrast to the mentioned studies, L-arginine supplementation positively modulates stressor-induced cognitive impairments and plasma and brain tissue biochemical changes in animal studies in which anxiety and depression are induced, as well as improves cognitive impairment in Alzheimer's disease model induced by type-2 diabetes,[33] improves anxiety in a stress model induced by electric shock,[34] and anxiotic signs induced by restraint stress and assessed by elevated plus-maze, anxiety-like behavior (EPM).[10,35] The use of NO donors or NOS inhibitors in experimental models has produced conflicting results, but also similar effects in the same study. Both L-arginine and L-NAME administered locally to the basolateral amygdala region reduced stress-induced anxiety and depression,[34] while L-arginine and low doses of L-NAME improved anxiety induced by restraint stress and assessed by EPM.[10] In addition, while the use of L-arginine alone did not cause any effect, it decreased the antidepressant effect of lithium when used with lithium, L-arginine supplementation improved aminophylline-induced anxiety-like behaviors, while anxiety level increased after L-NAME supplementation. However, L-NAME improved the number of seizures and mortality rate that developed as a result of aminophylline administration, whereas L-arginine had a negative effect. Considering all these reports, the fact that both NO precursor and inhibitor lead to the same result under the same conditions, and the interaction with different systems leads to unpredictable results suggest that NO is a neuromodulator that interacts with other factors such as stress factor, dose and duration of administration rather than a unique mechanism of action in the physiopathology of mood disorders such as anxiety and depression. The existence of bidirectional relationship between diabetes and depression, beyond the confusion of whether diabetes causes depression or depression causes diabetes, an important fact that should not be overlooked is that many psychological symptoms accompany diabetic individuals. Good management of depression and/or anxiety symptoms is clinically very important in the control of diabetes. The difficulty of preventing the complications of diabetes and the frequent overlook of the psychiatric disorders observed during diabetes cause these two comorbid conditions to worsen. Determining the underlying mechanisms of depression and/or anxiety states observed during diabetes will contribute to the explanation of the physiopathology of this comorbid condition. For this reason, many experimental animal studies are carried out for this purpose, but a clear answer has not been found yet, so it is thought that more comprehensive studies are needed in this area.[1,2,36]

CONCLUSION

Taken together, in this study, we demonstrated that STZ-induced diabetes caused both anxiety and depression in rats. Furthermore, AChE inhibition by donepezil had no effect on anxiety and depression levels in diabetic rats. An increase in the level of depression was observed in the L-arginine-treated group, while the non-specific NOS inhibitor L-NAME had no statistically significant effect. Our findings suggest that the depressive effects of L-Arginine may be related to the increase in ACh release by NO and the increase in the amount of ACh as a result of the effect of AChE inhibitor.

ETHICAL DECLARATIONS

Ethics Committee Approval: This study was carried out at Necmettin Erbakan University Experimental Medicine Researches and Application Center after obtaining Ethics Committee approval (2022/015).

Informed Consent: All patients signed the free and informed consent form.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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