Anticonvulsant and antioxidant effects of lamotrigine on pilocarpine-induced status epilepticus in mice

Kouhei Onishi, Tohru Kamida, Minoru Fujiki, Yasutomo Momii and Kenji Sugita

Objectives **The anticonvulsant and antioxidant effects of lamotrigine on status epilepticus (SE) are incompletely understood. We assessed these effects of lamotrigine on pilocarpine (Pilo)-induced SE in mice.**

Methods **Male C57BL/J6 mice were assigned to three groups: the control group, Pilo (400 mg/kg, s.c.)-induced SE (Pilo group) and lamotrigine (20 mg/kg, i.p.) treated (Pilo/lamotrigine group). The latency to SE of Racine's stage 3 or higher, the mortality rate within 2 h of Pilo administration, and the duration of SE until sacrifice were examined. Nitric oxide (NO), malondialdehyde and glutathione of oxidative stress biomarkers were detected in the hippocampus of the sacrificed animals in the above groups. NO was also detected in the cultured rat hippocampal neurons treated with 4 μM Pilo, Pilo+100 μM lamotrigine (Pilo/lamotrigine) and Pilo/lamotrigine+ N-methyl-D-aspartic acid (NMDA) receptor antagonist (10 μM MK-801, 3 μM ifenprodil) to examine the antioxidant effects of lamotrigine via non-NMDA-related pathways.**

Results **lamotrigine prolonged the latency to SE, the SE duration until sacrifice, and decreased the mortality rate in mice with Pilo-induced SE. Lamotrigine also decreased hippocampal concentrations of NO and malondialdehyde and increased the concentrations of glutathione in the SE model. Furthermore, there were significant differences in NO concentrations between groups of cultured rat hippocampal neurons treated with Pilo and Pilo/lamotrigine, and with Pilo/lamotrigine and Pilo/ lamotrigine+MK-801.**

Conclusion **Our findings suggest that lamotrigine exerts anticonvulsant and antioxidant effects on SE, but its antioxidant activity may not be fully exerted via NMDA-related pathways.** ***NeuroReport*** **34: 61–66 Copyright © 2022 The Author(s). Published by Wolters Kluwer Health, Inc.**

NeuroReport 2023, **34**:61–66

Keywords: lamotrigine, nitric oxide, oxidative stress, pilocarpine, status epilepticus

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Received 19 September 2022 **Accepted** 24 October 2022.

Introduction

Lamotrigine (3,5-diamino-6-2,3-dichlorophenyl-1,2,4-triazine) blocks activity at voltage- and use-dependent Na^+ channels in neurons, resulting in decreased glutamate release from presynaptic terminals [1]. Clinical evidence indicates that lamotrigine is effective against partial and secondarily generalized tonic-clonic seizures, as well as idiopathic generalized epilepsy. However, lamotrigine has failed to exhibit efficacy as an anti-status epilepticus (SE) drug in some experimental studies [2,3] and has not generally been used for SE in clinical settings.

Oxidative stress (OS) occurs when there is an imbalance in the redox environment resulting from excess reactive oxygen species (ROS), which in turn causes damage to lipids, proteins and DNA [4]. OS is involved in the development of various chronic and degenerative diseases such as cancer, inflammatory diseases and neurological diseases, including epilepsy [5]. Most recently, animal studies have addressed the role of OS and ROS generation in sustaining SE and generating spontaneous seizures in the context of chronic epilepsy [6,7]. Previous experimental studies have indicated that newer antiepileptic drugs (AEDs) exert antioxidant effects on OS in SE by measuring nitric oxide (NO), malondialdehyde and glutathione of oxidative stress biomarkers [8,9]. However, whether lamotrigine exhibits antioxidant properties depends on the experimental model and remains controversial [10–12]. Furthermore, no studies have reported the antioxidant effects of lamotrigine in an SE model except when used in combination with vitamin D [12].

Pilocarpine (Pilo) is a muscarinic cholinergic agonist that induces behavioral and electrographic seizures in rodents following systemic or intracerebral administration. Animals are motionless for 5–10 min after Pilo administration and subsequently display orofacial movements, salivation, eye-blinking, rearing, upper extremity clonus, falling and SE. Spontaneous remission of SE occurs within 5–6 h after Pilo administration [13]. Although previous animal studies have investigated the anticonvulsant effects of LTG on Pilo-induced SE, conclusive evidence is lacking [14], and no studies have reported the antioxidant effects of lamotrigine in such models.

The present study aimed to investigate the anticonvulsant effects of lamotrigine in Pilo-induced SE mice

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and to determine whether treatment with lamotrigine alters levels of the above-mentioned OS biomarkers in those mice hippocampus. Furthermore, we investigated the antioxidant effects of lamotrigine via N-methyl-D-aspartic acid (NMDA)-related pathways by measuring NO in in-vitro Pilo model with co-administered NMDA receptor antagonists [15,16] to eliminate other forms of OS in SE, such as hypoxia.

Materials and methods

Animals and experimental protocol

All experimental protocols were approved by the Oita University Ethical Review Committee (protocol number 192301). Male C57BL/J6 mice (60–90 days of age, body weight: 20–25 g; purchased from Charles River Laboratories, Japan) were housed at a controlled room temperature (24 ± 1 °C) under a 12/12-h light/dark cycle (light on at 8:00 a.m.). The room was maintained at constant humidity. Mouse food pellets and tap water were provided ad libitum between experimental procedures.

In Experiment 1, the animals were divided into three groups of 24 animals each. The control group included 24 mice treated with 1% Tween 80 (i.p.) dissolved in saline and no Pilo hydrochloride (control group). Among the 48 mice treated with Pilo (400 mg/kg, s.c.), 24 mice were treated with 1% Tween 80 (i.p.) dissolved in saline 30 min before Pilo treatment (Pilo group), whereas the remaining 24 were treated with both lamotrigine (20 mg/kg i.p.) and 1% Tween 80 dissolved in saline 30 min before Pilo treatment (Pilo/lamotrigine group). Lamotrigine was provided by Glaxo Smith Kline K.K. (Tokyo, Japan).

Mouse behavior was observed for up to 2 h after administration of Pilo. All mice developed SE of Racine's stage 3 or higher [17], some with severe tonic seizures leading to respiratory arrest and death. The latency to SE (min), the SE duration until sacrifice (min) and the mortality rate (%) were measured.

At the end of behavioral data collection, the mice that had respiratory arrest were immediately sacrificed via anesthetic overdose (350 mg/kg pentobarbital sodium, Henry Schein) before decapitation, while those that survived in the control, Pilo and Pilo/lamotrigine groups were sacrificed 2 h after administration of Pilo in the same way. The right hippocampus was quickly dissected out in toto, washed with ice-cold sodium phosphate buffer, weighed and stored on ice. The brains were further processed within 1 min of dissection, and biomarkers of OS were estimated on the same working day. Brain tissue was homogenized with 100 mg (w/v) sodium phosphate buffer. The homogenate was centrifuged at 3000 rpm for 15 min. Eight supernatants were used in each of the control, Pilo and Pilo/lamotrigine groups to estimate the activity of NO, malondialdehyde and glutathione. Assay systems for NO (FUJIFILM Wako Chemicals, Japan), malondialdehyde (Abcam, Japan) and glutathione disulfide (Dojindo, Japan) were used to quantify OS (Fig. 1).

Priel and Albuquerque [16] demonstrated the frequency of spontaneous postsynaptic currents in cultured rat hippocampal neurons following perfusion with Pilo (1 and 10 µM). Using the same technique described in their study, experiment 2 was performed to examine the antioxidant effects of lamotrigine via non-NMDA-related pathways. In this experiment, lamotrigine was co-administered with MK-801 (a selective noncompetitive NMDA receptor antagonist) or ifenprodil (a highly specific NR2B inhibitor) in cultured rat hippocampal neurons. Cultured primary rat hippocampal neurons (Invitrogen, Japan) were classified into four groups for the second experiment: a Pilo-control group (5 µl of saline 30 min before 4 µM of Pilo, $n = 5$ cultures), a Pilo/lamotrigine group (100 µM of lamotrigine pretreatment with coadministration of saline, $n = 5$ cultures), a Pilo/lamotrigine+MK-801 group (100 µM of lamotrigine pretreatment with coadministration of 10 µM of MK-801, $n = 5$ cultures) and a Pilo/lamotrigine+Ifenprodil group (100 µM of lamotrigine pretreatment with coadministration of 3 µM of ifenprodil, $n = 5$ cultures).

After 1 h, the cells were collected and centrifuged, and the amount of NO from the supernatant was measured and compared to investigate the effect of lamotrigine and its interactions with MK-801 and ifenprodil in cultured hippocampal neurons (Fig. 1).

Statistical analysis

The Mann-Whitney U-test was used to analyze the latency to SE and the SE duration until sacrifice, and the results are expressed as the mean ± standard error of the mean (S.E.M.). Chi-square tests were used to examine the mortality rate. The measured amounts of NO, malondialdehyde and glutathione were converted per gram of brain tissue and are expressed as the mean ± S.E.M. The Mann-Whitney U-test was used to analyze NO, malondialdehyde and glutathione values in each group. For all tests, P values less than 0.05 were considered statistically significant.

Results

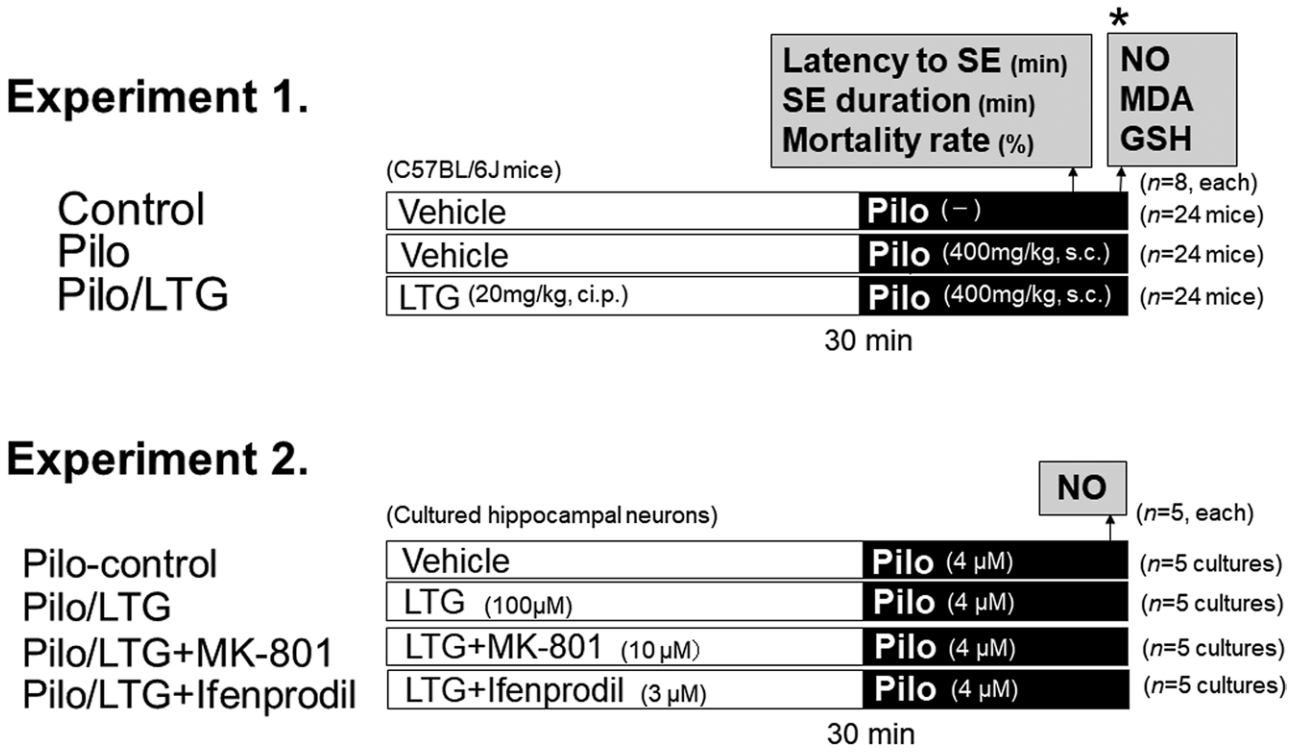
Effects of lamotrigine on seizure behavior

All mice treated with Pilo developed SE of stage 3 or higher within 1–2 h. The latency to SE and the SE duration until sacrifice were significantly longer, whereas the mortality rate was lower ($P = 0.06$), in animals pretreated with 20 mg/kg lamotrigine than in those treated with Pilo alone (Table 1).

Regulation of NO, malondialdehyde and glutathione concentrations with or without lamotrigine

Figure 2 illustrates the concentrations of NO, malondialdehyde and glutathione per gram of brain tissue from animals sacrificed immediately after death due to SE or 2 h after Pilo administration (Fig. 2a–c, respectively).

Fig. 1



Experimental protocol. Experiment 1: anticonvulsant and antioxidant effects of LTG in mice with Pilo-induced SE. Animals were prepared for behavioral testing, and the latency to SE (min), the SE duration until sacrifice, and the mortality rate (%) were measured after Pilo injection. Following behavioral testing, the concentrations of oxidative stress biomarkers (NO, MDA, and GSH) were measured to investigate the effect of LTG (Pilo/LTG, Pilo, and control groups, $n=24$ each). *NO, MDA and GSH concentrations in the right hippocampus were immediately measured in mice that had respiratory arrest were immediately sacrificed via anesthetic overdose before decapitation, while those that survived in the control, Pilo and Pilo/LTG groups were sacrificed 2 h after administration of Pilo in the same way. Experiment 2: antioxidant effects of LTG via non-NMDA-related pathways were investigated by co-administering LTG with MK-801, a selective noncompetitive NMDA receptor antagonist, or ifenprodil, a specific NR2B inhibitor. Cultured rat hippocampal neurons with Pilo-induced firing were classified into four different pretreatment groups. NO levels were measured 1 h after Pilo administration. GSH, glutathione; LTG, lamotrigine; MDA, malondialdehyde; NMDA, N-methyl-D-aspartic acid; NO, nitric oxide; Pilo, pilocarpine; SE, status epilepticus.

Table 1 The effects of lamotrigine on the latency of status epilepticus (SE) to Racine's stage 3 or higher, the SE duration until sacrifice, and the mortality rate in pilocarpine-induced status epilepticus mice

Drugs	Latency to SE (min)	SE duration until sacrifice (min)	Mortality rate (%)
Pilo (n=24)	14±2.1	32±8.1	79.2
Pilo+LTG (n=24)	40±9.6[a]	62±12[a]	50.0

LTG, lamotrigine; SE, status epilepticus.
[a]$P<0.05$ vs. Pilo group, Mann-Whitney U-test.

NO and malondialdehyde concentrations per gram of brain tissue were significantly higher in the Pilo group than in the control group ($P<0.05$; Fig. 2a,b), while they were significantly lower in the Pilo/lamotrigine group than in the Pilo group ($P<0.05$; Fig. 2a,b). In contrast, glutathione concentrations per gram of brain tissue were significantly lower in the Pilo group than in the control group ($P<0.05$; Fig. 2c), whereas they were significantly higher in the Pilo/lamotrigine group than in the Pilo group ($P<0.05$; Fig. 2c).

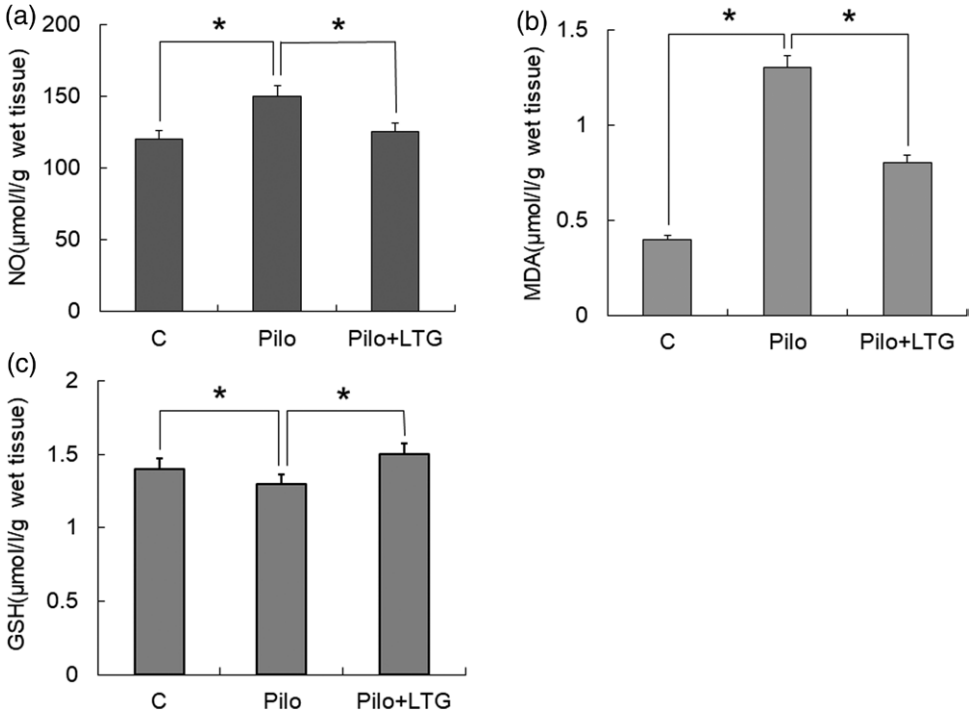
Effects of coadministration with MK-801 or ifenprodil

Administration of lamotrigine decreased NO concentrations in cultured rat hippocampal neurons with Pilo-induced firing, relative to concentrations observed in the Pilo-control group ($P<0.05$; Fig. 3). Furthermore, there was a significant difference in NO concentrations between the Pilo/lamotrigine and Pilo/lamotrigine+MK-801 groups ($P<0.05$; Fig. 3).

Discussion

In this study, we demonstrated that lamotrigine exerts anticonvulsant effects on Pilo-induced SE in mice by prolonging the latency to SE, the SE duration until sacrifice, and decreasing the mortality rate. Our findings also indicated that lamotrigine exerts antioxidant effects on Pilo-induced SE by decreasing NO and malondialdehyde concentrations and increasing glutathione concentrations.

Fig. 2



Quantitative analyses of NO, MDA, and GSH concentrations in the hippocampus as biomarkers of oxidative stress after Pilo administration with or without LTG. NO and MDA concentrations per gram of right hippocampal tissue were significantly higher in the Pilo group than in the control group, while they were significantly lower in the Pilo/LTG group than in the Pilo group (a,b). GSH concentrations per gram of unilateral hippocampal tissue were significantly lower in the Pilo group than in the control group, while they were significantly higher in the Pilo/LTG group than in the Pilo group. Each value represents the mean ± S.E.M. ($n=24$). Significantly different from the control group or the Pilo group using Mann-Whitney U-test (*$P<0.05$). GSH, glutathione; LTG, lamotrigine; MDA, malondialdehyde; NO, nitric oxide; Pilo, pilocarpine.

Furthermore, there were significant differences in NO concentrations between Pilo/lamotrigine and Pilo/lamotrigine+MK-801 groups in cultured rat hippocampal neurons with Pilo-induced firing, indicating that lamotrigine may not exert sufficient antioxidant effects via NMDA-related pathways.

Anticonvulsant effects of lamotrigine on Pilo-induced SE

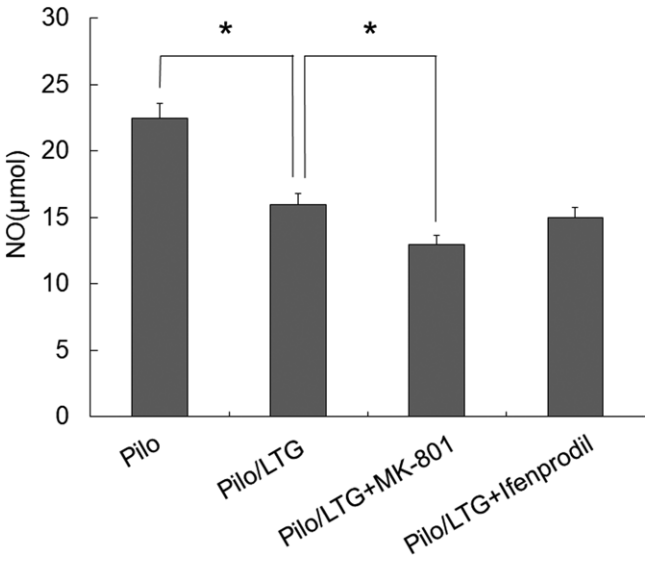
There are some clinical cases that reported the anticonvulsant effect of lamotrigine in patients with SE, although the mechanism of action remains to be clarified [18,19]. However, none of the experimental studies have been unable to confirm the anticonvulsant effects of lamotrigine on SE [2,3]. To our knowledge, the current study is the first experimental investigation to demonstrate the anticonvulsant effects of lamotrigine on SE. Our study differs from previous work in terms of the method used to induce SE and the timing of lamotrigine administration.

Some evidence suggests that Pilo-induced seizures are initiated via muscarinic receptors and further mediated via NMDA receptors [20]. Maintenance of SE may involve not only the activation of NMDA receptors [21] but also astrocytic Ca^{2+}-dependent glutamate release [22] and a loss of gamma aminobutyric acid (GABA)ergic synaptic inhibition [23]. Lamotrigine stabilizes nerve cell membranes by suppressing Na^{+} channels [24] and acting on Ca^{2+} channels, thereby blocking Ca^{2+} influx [25]. Lamotrigine may suppress the excessive release of excitatory neurotransmitters such as glutamate by stabilizing the cell membrane and decrease $GABA_A$ receptor-mediated synaptic transmission via direct and/or indirect effects on presynaptic Ca^{2+} influx [26]. These mechanisms may explain the anticonvulsant effects on SE of lamotrigine. Moreover, for lamotrigine to exert anticonvulsant activity against Pilo-induced SE, sufficient increases in blood concentration are required before Pilo administration, and it may take at least 30 min to observe these effects, as shown in our experiments.

Antioxidant effects of lamotrigine on Pilo-induced SE

Freitas *et al.* [7] have reported significant increases in lipid peroxidation and nitrite concentration accompanied by decreases in glutathione content in rats with Pilo-induced SE. Our data regarding the hippocampal redox environment after Pilo-induced SE are in accordance with those of Freitas *et al.*, [7] indicating that lamotrigine alone exerted antioxidant effects in our animal model by restoring NO, malondialdehyde and glutathione to levels near the control values. However, in contrast to our results, Mahfoz *et al.* [12] were unable to demonstrate the

Fig. 3



Effects of co-administering LTG with MK-801 or ifenprodil on NO concentrations in cultured rat hippocampal neurons with Pilo-induced firing. Administration of LTG decreased NO concentrations in cultured rat hippocampal neurons, relative to concentrations observed in the Pilo-control group. Furthermore, there was a significant difference in NO concentrations between the Pilo/LTG and Pilo/LTG+MK-801 groups. Each value represents the mean ± S.E.M. (n=5 cultures). Significantly different from the Pilo group or the Pilo/LTG group using Mann-Whitney U-test (*P<0.05). LTG, lamotrigine; NO, nitric oxide; Pilo: pilocarpine.

antioxidant effects of lamotrigine alone on Pilo-induced SE. This discrepancy may have been due to differences in the methods of drug administration between the two studies.

Overactivation of NMDA receptors is known to trigger excessive calcium influx, resulting in excessive generation of free radicals, including ROS and reactive nitrogen species such as NO [27]. The antioxidant activity of lamotrigine against Pilo-induced SE, as well as its anticonvulsant activity, may be mediated by the NMDA receptor pathway. However, our in-vitro data suggest that its antioxidant activity may not be fully exerted via NMDA-related pathways. Other studies have also reported antioxidant activities of lamotrigine that are not mediated by the NMDA receptor pathway. Lizasoain *et al.* [10] demonstrated that lamotrigine directly inhibits the synthesis of NO in rat forebrain slices. Arora *et al.* [28] reported that the antioxidant effects of lamotrigine may be associated with the inhibition of glutamate-mediated excitotoxicity via N-malondialdehyde receptors. Other in-vitro studies have reported that lamotrigine exerts antioxidant activity via suppression of rotenone and 1-methyl-4-phenylpyridinium, leading to ROS formation and glutathione depletion [29]. In this study, the antioxidant effects of lamotrigine via non-NMDA-related pathways are unknown. A control group with Pilo+MK-801 will be needed for additional comparison in our in-vitro study.

Acknowledgements

The authors are grateful to Glaxo Smith Kline K.K. for providing lamotrigine.

This work was supported by a Grant-in-Aid for Scientific Research (C) [grant number 20K12626].

T.K. and M.F. designed the research paradigm; K.O., T.K., M.F., Y.M. and K.S. performed the research investigations; K.O., T.K. and M.F. analyzed the data and K.O., T.K. and M.F. wrote the article.

The data that supprt the findings of this study are openly available.

Conflicts of interest

There are no conflicts of interest.

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