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Evaluation of acetylcholinesterase activity and behavioural alterations induced by ketamine in an animal model of schizophrenia

Zugno AI, Matos MP, Canever L, Fraga DB, De Luca RD, Ghedim FV, Deroza PF, de Oliveira MB, Pacheco FD, Valvassori SS, Volpato AM, Budni J, Quevedo J. Evaluation of acetylcholinesterase activity and behavioural alterations induced by ketamine in an animal model of schizophrenia.

Objective: Cognitive deficits in schizophrenia play a crucial role in its clinical manifestation and seem to be related to changes in the cholinergic system, specifically the action of acetylcholinesterase (AChE). Considering this context, the aim of this study was to evaluate the chronic effects of ketamine in the activity of AChE, as well as in behavioural parameters involving learning and memory.
Methods: The ketamine was administered for 7 days. A duration of 24 h after the last injection, the animals were submitted to behavioural tests. The activity of AChE in prefrontal cortex, hippocampus and striatum was measured at different times after the last injection (1, 3, 6 and 24 h).
Results: The results indicate that ketamine did not affect locomotor activity and stereotypical movements. However, a cognitive deficit was observed in these animals by examining their behaviour in inhibitory avoidance. In addition, an increase in AChE activity was observed in all structures analysed 1, 3 and 6 h after the last injection. Differently, serum activity of AChE was similar between groups.
Conclusion: Chronic administration of ketamine in an animal model of schizophrenia generates increased AChE levels in different brain tissues of rats that lead to cognitive deficits. Therefore, further studies are needed to elucidate the complex mechanisms associated with schizophrenia.

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Keywords: acetylcholinesterase, ketamine, memory, schizophrenia

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Significant outcomes

- Ketamine induces the increase of acetylcholinesterase (AChE) activity.
- Ketamine induces impairment of memory.

Limitations

- The implementation of additional behavioural and biochemical tests (pre-pulse inhibition and expression of cholinergic receptors, for example), could further reinforce our conclusions, or could open up a different line of discussion.

Introduction

Schizophrenia is a psychiatric disorder that affects ~20 million people worldwide, leading to serious professional and social restrictions for patients (1). Cognitive improvement has been the most important challenge in schizophrenia treatment and many other diseases as well. Schizophrenia was firstly called 'dementia praecox' by Kraepelin (2), owing to its onset in young adults, in contrast to the more usual 'elderly dementia'. The current pharmacological treatment for schizophrenia has succeeded in diminishing psychotic symptoms, but not cognitive deficits. Actually, antipsychotics ameliorate the natural history of schizophrenia (3), but their benefits are not enough to provide a better life quality and restore the cognitive function in similar patterns to non-schizophrenic controls (4).

It was observed in animal models that changes in cortical glutamatergic function are related to dysfunction in subcortical dopaminergic neurotransmission, which also affects the action of cholinergic system in schizophrenic population (5–8). In patients, these abnormalities in glutamatergic neurotransmission are linked to memory deficits, as it is known that the excitation of glutamate is essential for the formation of different types of memory (9).

Acetylcholine (ACh) is a neurotransmitter present in both the peripheral nervous system and central nervous system of many organisms. Cholinergic neurons form a neurotransmitter system, from the brainstem and basal forebrain, which projects axons to many areas of the brain. ACh is involved in synaptic plasticity, specifically in learning and short-term memory (STM) (10,11). ACh has been shown to enhance the amplitude of synaptic potentials following long-term potentiation in many regions, including the dentate gyrus, CA1, piriform cortex and neocortex. This effect can occur either by enhancing *N*-methyl-D-aspartate receptor (NMDAR) expression or indirectly by suppressing adaptation (12,13). In addition, epidemiological surveys have shown that schizophrenic patients make more use of cigarettes (tobacco) than the general population and these patients present more severe positive symptoms of the disease, indicating that ACh receptors may be related to this disorder (14,15).

AChE is the enzyme that breaks ACh in the synaptic cleft in choline and acetate, being choline reuptake and recycled presynaptically (16). The AChE inhibitors are mainly used in the treatment of Alzheimer's disease. Although some efficacy on cognitive deficits, they do not change the course of the disease, and its role in the treatment of the disease is unclear (17). In addition, a recent meta-analysis has shown that que-specific cognitive deficits (memory, motor speed and attention) of patients with schizophrenia and schizoaffective disorder are responsive to rivastigmine, donepezil and galantamine (currently used AChE inhibitors in Alzheimer's disease), the adjunctive therapy. However, confirmatory studies are needed to determine the clinical utility of this treatment strategy (18,19).

The model of ketamine is one of the most widely accepted models for behavioural and biochemical alterations in animals, similar to human schizophrenia (20). Ketamine is a non-competitive antagonist of NMDAR, an ionotropic glutamate receptor. The NMDAR hypofunction is a well-established alteration in schizophrenia and is involved in memory function (21). Considering this information, we opted to use this animal model to test the hypothesis that ketamine alters AChE activity in a short period of time. Moreover, our proposal was also to investigate what kind of behavioural changes may be seen 24 h after the last injection, as there is evidence that behavioural changes may be secondary to biochemical alterations.

Materials and methods

The experiments were conducted in accordance with the Brazilian Society for Neuroscience and Behaviour's recommendations for animal care, after the approval of the Ethics Committee in Animal Usage of the Universidade do Extremo Sul Catarinense (protocol number 96/2009). The entire experiment was conducted in the laboratory of neurosciences at the Universidade do Extremo Sul Catarinense.

Animals

Adult male Wistar rats weighing 250–300 g were obtained from our breeding colony. The animals were housed in acrylic cages (five animals per cage) with food and water available *ad libitum* and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). Two groups of animals (saline or ketamine) were destined to behavioural tests. Specifically, 12 animals per group were intended to assess the locomotor activity and stereotypy. An independent amount of animals (12 per group) was used for memory evaluation on inhibitory avoidance. Similarly, for the evaluation of object recognition, 12 animals per group were used. Therefore, a total of 72 animals were used for all behavioural tests. For the analysis of AChE activity in different times, independent groups were selected and killed 1, 3, 6 or 24 h after the last injection. The brain structures such as the prefrontal cortex, striatum and

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hippocampus were dissected and rapidly frozen at −80°C until the biochemical analysis. For this procedure, a number of five animals per group were necessary.

Animal model of schizophrenia

Schizophrenia symptoms were induced by chronic sub-anaesthetic doses of ketamine at 25 mg/kg for 7 days. This dose induces hyperlocomotion and stereotypy (20,22). Specifically, the animals received ketamine intraperitoneally once a day during 7 days. A duration of 24 h after the last injection, the animals were destined to behavioural evaluations, whereas an independent group of animals was killed 1, 3, 6 and 24 h after the last injection for the evaluation of AChE activity.

Behavioural tests

Open-field task. The open-field task was performed in a 50 × 25 × 50 cm arena. Locomotor activity was monitored using a computerised system (Activity Monitor; Insight Laboratory Equipments, Ribeirão Preto, SP, Brazil). This equipment monitored the locomotor activity measuring the travelled distance by each rat into blocks for 5 min. For the analysis, the total distance of each block was measured in both groups (saline or ketamine) over a period of 60 min.

Stereotypy. Stereotypy is defined as rapid, repetitive, frontward movements (23–25). This parameter was analysed along with locomotor activity. Stereotypy is considered by the software as an unstable movement at any time when repetitive movements are recorded in sequential readings without alterations in the animal's mass centre. This evaluation was included to guarantee that stereotypy was no longer present by the time the animal's memory was evaluated, and therefore any alterations observed in memory tasks were not to be attributed to the ketamine's acute effects, but to the long-lasting impairments caused by chronic administration of the drug.

Inhibitory avoidance. The inhibitory avoidance evaluation was initiated 24 h after the last injection. The apparatus consisted of an acrylic box whose floor is constructed from parallel stainless steel bars, with a platform placed against the left wall of the box (26,27). In the training session, rats were placed on the platform and we measured their latency to step down with all four paws. Immediately after step down from the platform, the animals received a 0.4 mA footshock (electrical shock induced through the feet) for 2 s.

In the test session, animals were again placed on the platform and had their latency to step down on the grid measured, except that no footshock was given. Latency is a classic parameter for memory retention tasks. Test sessions for working memory were conducted immediately after training (5 s) (28). The given interval between training and test sessions was 1.5 h to evaluate STM (29,30) and 24 h to long-term memory (LTM) (30,31).

Object recognition test. The task was performed in the open-field arena and conducted as protocol described elsewhere (31–33). Habituation was initiated 24 h after last ketamine injection: animals were placed in the left posterior corner of the apparatus and allowed to explore environment for 5 min. No objects were present during the habituation phase.

On the second day, 24 h after habituation, the training session was conducted. Animals were again placed in the apparatus, where two identical objects (A1 and A2) were located and the exploration time of each object was measured during a total period of 5 min.

During the same session, 1 h and 30 min after training, the animal's STM was measured: the animals were placed in the apparatus in the presence of the first familiar object (A1) and the new object to be recognised (B). Again, the time animals took to explore each object was measured and recorded.

On the third day, 24 h after training, the animal's LTM was evaluated following the same procedure applied for STM; however, the object B was exchanged for object C (which is also different to object A).

AChE activity

The activity test for this enzyme was conducted according to the method of Ellman et al. (34). The hydrolysis of ACh was assessed in a concentration of 0.8 mM in 1 ml of a solution containing 100 mM phosphate buffer (pH 7.5) and 1.0 mM DTNB. A volume of 50 μl of each sample was added to the solution and pre-incubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB to 412 nm for 2–3 min at intervals of 30 s to 25°C. The samples were evaluated in duplicates.

Protein levels

Protein levels were determined using the Lowry (35) method, with bovine serum albumin used as a standard.

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Statistical analysis

Data from AChE activity, open-field task and stereotypical movements were analysed by the Student *t*-test for unpaired samples. Training-test session latency differences and object recognition were assessed by the Wilcoxon test, followed by individual Mann–Whitney *U*-test. All analyses were performed using the statistical package for the social sciences (SPSS) software. A value of $p < 0.05$ was considered as statistical significance.

Results

Locomotor activity (Fig. 1) and stereotypical movements (Fig. 2) evaluated 24 h after the last ketamine injection showed no significant difference when compared with the control group. Furthermore, the total covered distance travelled by the groups was similar (data not shown). Thus, we can suggest that results found in the following memory tasks are more likely a consequence of the long-lasting impairments caused by chronic ketamine administration, rather than the drug's acute effect.

Working memory tested in the inhibitory avoidance task was shown to be impaired, as ketamine-treated animals showed significantly lower latency to step down from the platform, when compared with the saline group (Fig. 3). The same occurred when the STM and LTM were evaluated using the same task 1.5 and 24 h after training (Fig. 4). In both sets of tests, a significantly lower latency was seen within the ketamine-treated animals, indicating poorer memory acquisition when compared with saline-treated animals.

We also evaluated the animals STM and LTM memories in the object recognition task. Again, 1, 5 and 24 h after-training trials, animals that were chronically treated with ketamine showed memory impairment, with significantly lower recognition indexes, when compared with control group (Fig. 5).

The activity of the enzyme AChE was increased in all brain structures analysed, which were: the striatum, hippocampus and prefrontal cortex at time intervals of 1, 3 and 6 h after the last injection of ketamine. The same result was not observed in animals that were analysed 24 h after the last dose of the same drug (Fig. 6).

Regarding the evaluation of AChE in serum, in search for a possible peripheral marker of the disorder, there were no significant differences between ketamine-treated animals and the control group (Fig. 6).

Discussion

Cognitive impairment is a characteristic symptom of schizophrenia and it is present even in the prodromal

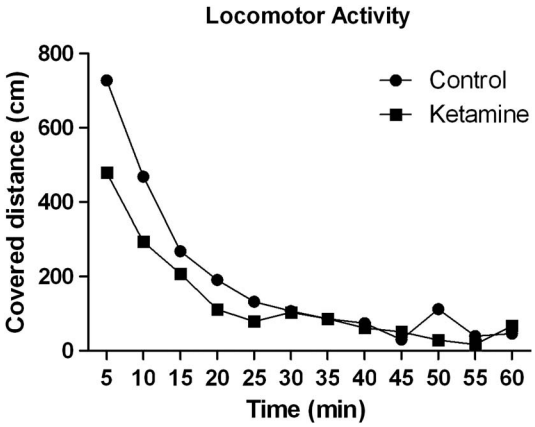

Fig. 1. Locomotor activity was monitored using a computerised system. The locomotor activity measured the distance travelled by each rat into blocks of 5 min. The data were organised in SPSS version 17.0, with a confidence interval of 95% and significance level $\alpha = 0.05$. $p < 0.05$.

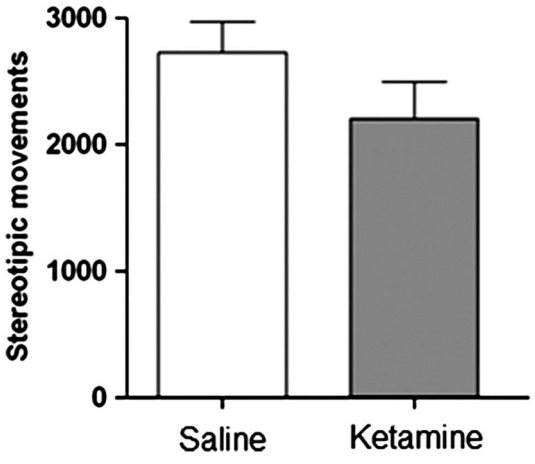

Fig. 2. Stereotypical movements were analysed along with locomotor activity and were evaluated 24 h after the last ketamine injection and compared with the control group. The data were organised in SPSS version 17.0, with a confidence interval of 95% and significance level $\alpha = 0.05$ ($p < 0.05$).

phase of the disorder (36–38). For schizophrenic patients, memory deficits have a major influence on recovery, social adjustment and functional aspects of daily lives (39–41). Thus, animal models addressing schizophrenia's cognitive impairments can contribute to our better understanding and possible future treatments.

The chronic administration of ketamine cause long-lasting cognitive deficits in rodents, even after interrupting ketamine administration. Thus, this seems to be a good animal model for a chronic disease as schizophrenia, especially for 'negative symptoms'. In a study by Chatterjee et al. (42) these behavioural alterations persisted at least for 10 days, after the withdrawal of ketamine treatment. An *in vitro* study

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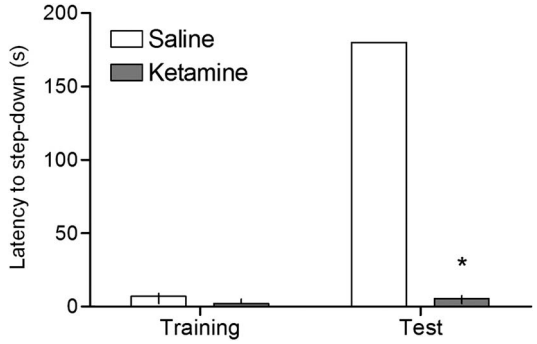

Fig. 3. Evaluation of working memory in both groups (saline or ketamine) was taken 24 h after the last injection. In the training session, rats were placed on the platform and we measured their latency to step down with all four paws. Immediately after step down from the platform, the animals received a 0.4 mA footshock (electrical shock induced through the feet) for 2 s. To check whether the differences between the paired groups was significant, a Student's *t*-test was used, considering as significant differences in those with $*p < 0.05$, different from saline.

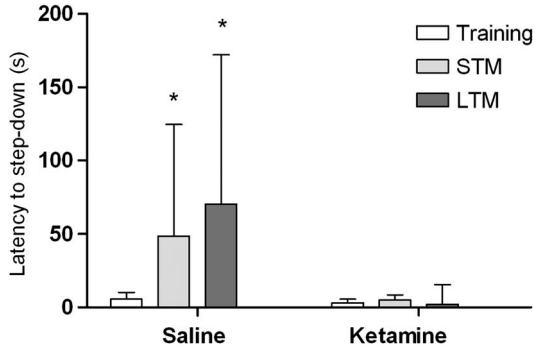

Fig. 4. The short-term memory (STM) and long-term memory (LTM) were evaluated using the inhibitory avoidance task 1.5 and 24 h after training. A significantly lower latency was seen within the ketamine group, indicating poorer memory acquisition when compared with saline-treated animals. The data were organised in SPSS with a confidence interval of 95% and significance level $\alpha = 0.05$ ($*p < 0.05$, different from training).

showed that sub-anaesthetic concentrations of ketamine, while not affecting cell survival, may still impair neuronal morphology and thus might lead to dysfunctions of neural networks (43).

Our results show that chronic sub-anaesthetic administration of ketamine impairs the rat's immediate memory, as well as its STM and LTM. Similar results from our group were previously published (44). However, the novelty here is the result of object recognition test. Furthermore, our findings corroborate previous studies that have shown that ketamine (at sub-anaesthetic or anaesthetic doses) negatively affects the rat's performance in different memory task by the blockade of NMDARs (33,45–47).

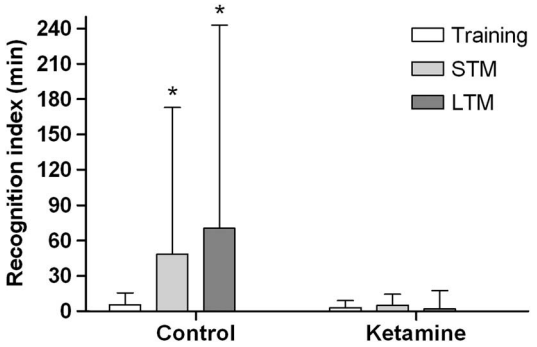

Fig. 5. The object recognition task was performed in the open-field arena. Durations of 1.5 and 24 h after training, animals chronically treated with ketamine showed memory impairment, with significantly lower recognition indexes, when compared with control group. To check whether the differences between the paired groups was significant the Student's *t*-test was used, considering as significant differences in those with $*p < 0.05$ (different from training). LTM, long-term memory; STM, short-term memory.

The relationship involving ACh and cognitive functions is well described in current literature (28,48–51). Several studies have demonstrated the association between augmented ACh release and improvement in learning and memory, whereas impairments in these cognitive functions are linked to a decline in ACh release. This occurrence was observed in brain structures such as the hippocampus, nucleus accumbens, insular cortex, neocortex and amygdala (48). It is important to highlight that the memory impairment seen 24 h after the injection of ketamine was not accompanied by changes in the activity of the enzyme AChE at that time. It suggests that the behavioural changes observed in inhibitory avoidance can be a secondary effect of increased AChE in 1, 3 and 6 h after ketamine treatment. In addition, it can possibly be a result of modifications in other neurotransmitter systems induced by ketamine (e.g., a modulatory effect on the monoamines) (52). Furthermore, the normalisation of AChE activity 24 h after the last injection of ketamine suggests the return of the balance in the levels of ACh in the synaptic cleft. However, the reduction of ACh induced by ketamine may initially induce an internalisation of cholinergic receptors in the postsynaptic membrane, causing a late cognitive effect. Therefore, our results may be because of this fluctuation of cholinergic markers (52).

There are several regions of the brain that can be affected during the course of schizophrenia, leading to its distinctive symptoms. Whereas paranoia and hallucinations can be precipitated by an abnormal function in the basal ganglia, agitation is highly related to the limbic system and an impaired hippocampal formation, inducing in learning and memory deficits. Defects in the frontal lobe are

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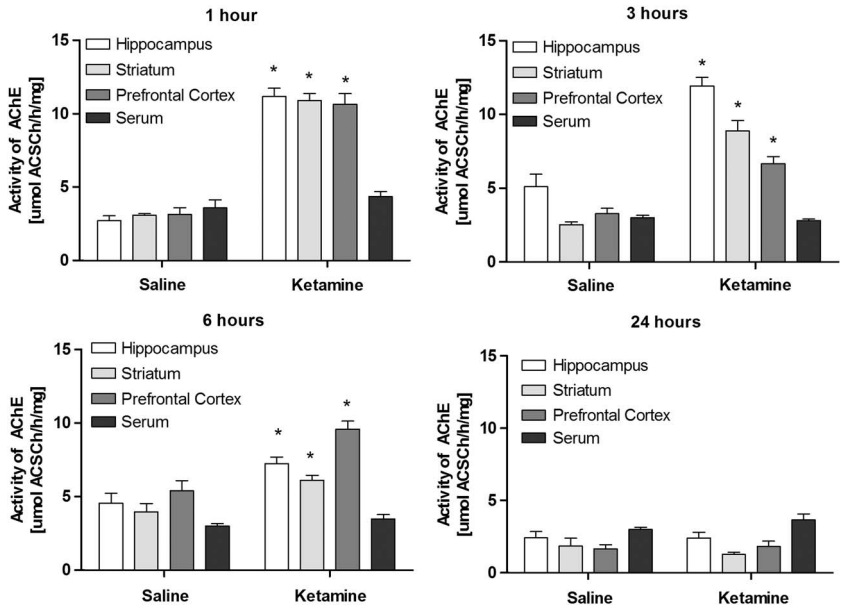

Fig. 6. The activity of the enzyme acetylcholinesterase (AChE) was increased in all brain structures analysed in 1, 3 and 6 h; however, no significant difference was observed in 24 h between ketamine-treated animals and the control group. To check the differences between the structures treated with saline or ketamine, the Student's *t*-test was used, considering as significant differences in those with $*p < 0.05$.

associated with problems involving executive functions, for example, planning and organisation (53).

Studies concerning the neurochemistry of the cognitive functions have emphasised the connection between the cholinergic and glutamatergic systems. Cholinergic activity in the cortex produces a complex combination of inhibitory and excitatory effects, which interfere with glutamatergic signalling via NMDARs (49). According to Colgin et al. (54), regarding the pathophysiology of schizophrenia, cholinergic signals support the excitatory activity of glutamate in the encoding of memory in hippocampus, and thus ACh acts by modulating the glutamatergic action. In this context, in our present study, an increased activity of AChE was observed in rats that received ketamine 1, 3 and 6 h after the last injection. These results are probably subsequent to changes in glutamatergic transmission by ketamine (55). γ-Aminobutyric acid and AChE in rodent cortical neurons coexist (56), and there can be simultaneous release of glutamate and ACh from basal forebrain neurons as well (57). Thus, modifications in AChE can reflect changes in other neurotransmitter systems, not only in cholinergic. Our study is limited by the lack of specificity of the AChE activity. Because the activity of an enzyme can be modified by several mechanisms, it is not possible to infer whether there were changes in substratum concentrations, enzyme production or environmental (intra- or extracellular) conditions (58).

One of the reasons suggested for the ACh decrease in schizophrenia is the alteration of the nicotinic receptors in this disorder. This hypothesis is sustained by studies indicating that schizophrenic patients make a significantly larger use of tobacco when compared with general population (14,59–61). In addition, studies with schizophrenic patients suggest that the ones that make high use of tobacco present more severe positive symptoms, while the use of tobacco is also associated with a decrease of the negative symptoms (61–63).

We also analysed AChE in the animal's serum after treatment with ketamine, but we found no statistical relevance when comparing with the control group. It confirms that schizophrenia is still a disorder of primarily clinical diagnosis (22,64), corroborating with the absence of a peripheral marker. Therefore, further studies correlating the drugs already used in mental disorders in an attempt to unravel the intricate pathophysiology mechanisms of schizophrenia and aiming for a better control of the disorder are needed.

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Authors' contributions

A.I. Zugno, J. Quevedo, D.B. Fraga and M.P. Matos: designed the study and wrote the protocol;

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M.P. Matos, L. Canever, D.B. Fraga, R.D. De Luca, F.V. Ghedim, P.F. Deroza and M.B. Oliveira: carried out the protocol; A.I. Zugno, F.D. Pacheco, S.S. Valvassori, A.M. Volpato and J. Budni: managed the literature searches and statistical analyses; D.B. Fraga, J. Budni, F.D. Pacheco and A.M. Volpato: wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Declaration of Interest

The authors declare that they have no conflict of interest.

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