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Potential PDE4A inhibition-mediated neuroprotective effects of psoralidin

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Abstract. – **OBJECTIVE:** This study aimed to investigate the effect of psoralidin, a natural phenolic coumarin compound, on MK-801-induced neurotoxicity that may cause Alzheimer's disease and to determine the phosphodiesterase (PDE)-related molecular mechanism of action.

MATERIALS AND METHODS: In this study, neurotoxicity was performed using the MK-801 in the HT-22 cell line. The effects of compounds on the proliferation of HT-22 cells were determined by Real-Time Cell Analysis (RTCA). After measuring the total protein concentration, the PDE4A protein level was determined using the Western blot method.

RESULTS: Psoralidin (100, 200, 400 µM) has been shown to have a neuroprotective effect against MK-801-induced neurotoxicity, as indicated by Real-Time Cell Analysis. In HT-22 cells, the half maximal effective concentration (EC_{50}) value of psoralidin was calculated to be 230.4 µM, IC_{50} value of MK-801 was calculated to be 62.4 µM at 24 hours. It has been determined that psoralidin (200, 400 µM) inhibits PDE4A by using the Western blot method.

CONCLUSIONS: This research uncovers that psoralidin has neuroprotective effects in MK801-associated accumulation of the excitatory amino acid glutamate neurodegeneration and Alzheimer's disease.

Key Words:
Alzheimer's disease, Neuroprotective, Psoralidin, HT-22 cell line, PDE4A.

Introduction

Alzheimer's disease (AD) is characterized by progressive and eventual loss of multiple cognitive functions and accounts for a high percentage of dementia cases[1,2]. AD is a synaptic dysfunction encompassing molecular, cellular, and cognitive disorders. AD pathology includes tau-containing neurofibrillary tangles, amyloid beta (Aβ)-containing plaques, activated glia, and neuronal cell death[3].

Phosphodiesterase 4A is a protein encoded by the PDE4A gene in humans. PDE plays an essential role in many important physiological processes by regulating the cellular concentration of cyclic adenosine monophosphate (cAMP)[4]. The benefit of blockade by selective PDE inhibitors can be seen in various brain pathologies, such as depression, schizophrenia, AD, and ischemia. Recent studies have focused on the effects of phosphodiesterase enzyme inhibitors (PDEIs) on neurological and psychiatric diseases[5,6].

Psoralidin is a natural phenolic coumarin compound that is a derivative of coumestane and is found in the seeds of *Psoralea corylifolia*[7-9]. Psoralidin exhibits various biological activities, such as anticancer, antioxidant, antibacterial, antidepressant, and regulation of insulin signaling[10]. Intracellular changes caused by psoralidin include synaptic modulation, activation of NMDA receptors, and stimulation of extracellular calcium influx[11].

MK-801 is an extremely potent and selective, non-competitive N-methyl-D-aspartate (NMDA) glutamate receptor antagonist that affects the NMDA receptor, a glutamate receptor with ion channel properties[12]. Chronic, mild activation of NMDA receptors ultimately leads to neurodegeneration, an effect termed chronic excitotoxicity[13]. Excessive accumulation of glutamate (GLU) in the extracellular space produces toxic effects for central mammalian neurons. The glutamatergic system in general, and NMDA receptors in particular, may play a significant role in the execution of synaptic dysfunction and neuronal death triggered by Aβ in AD[13].

This study aimed to investigate the effect of psoralidin on MK-801-induced neurotoxicity and to determine the PDE-related molecular mechanism of action.

Materials and Methods

HT-22 mouse hippocampal neuronal cell lines (SCC129) were purchased from the Merckmillipore (Burlington, MA, USA). Dulbecco's Modi-

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fied Eagle Medium (DMEM), β-mercaptoethanol, Tween-20 (P5927), and 100 U/ml penicillin-100 μg/ml streptomycin were purchased from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Applichem (Darmstadt, Hessen, Germany). Fetal bovine serum (FBS), BCA protein assay kit, and West-Pico electrochemiluminescence (ECL) reagents were purchased from Thermo Fischer Scientific (Pierce™) (Waltham, MA, USA). The 96-well E-plate was purchased from ACEA Biosciences (San Diego, CA, USA). Immun-Blot PVDF Membrane (162-0177) was purchased from Bio-Rad (Hercules, CA, USA). Nonfat dry milk (70166) was purchased from Fluka (Muskegon, Mich, USA). Radioimmunoprecipitation assay (RIPA) lysis buffer (sc-24948), psoralidin (sc-202779), and MK-801 (sc-203137) were purchased from Santa Cruz (Heidelberg, Baden-Württemberg, Germany). β-Actin Ab (4967S) was purchased from Cell Signaling (Danvers, MA, USA). PDE4A Antibody (16226-1-AP) and Goat anti-rabbit IgG HRP conjugate (SA00001) were purchased from Proteintech (Rosemont, IL, USA).

Cell Cultures

The HT-22 mouse hippocampal neuronal cell line was propagated in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. Cells were incubated at 37°C in 95% air and 5% CO_2.

Real-Time Cell Analysis (RTCA)

The activity of these compounds was monitored with the xCELLigence Real-Time Cell Analyser (RTCA) system, which measures electrical impedance and exhibits cell index. The optimum seeding concentration of HT-22 cells was determined by profiling, and then, the cells were seeded on E-plate at 20,000 cells per well. Real-time impedance measurement enables precise detection of cellular status from low cell counts to confluence as frequent measurements are made between biosensors. Cells were treated at specified concentrations (10, 50, 100, 200, 400 μM) with MK-801. Subsequent analysis was performed with MK-801 [200 μM in combination with psoralidin (10, 50, 100, 200, 400 μM)]. The experiments were run for about 60 hours. According to the manufacturer's instructions (ACEA Biosciences, San Diego, CA, USA), cell attachment, spreading, and proliferation should be monitored every 30 minutes using the RTCA instrument. However, in this study, CI values were measured every 15 minutes to enable sufficiently frequent analysis.

The RTCA-integrated software enabled the calculation of EC_{50} (half-maximal effective concentration) and IC_{50} (half-maximal inhibitory concentration) values.

Western Blotting

1 x 10^6 cells were seeded in 6 well plates and incubated. After 24 hours of incubation with the molecules, lysates were obtained by treatment with RIPA lysis buffer containing protease inhibitors. 40 μg protein lysates were heated in a sample buffer containing β-mercaptoethanol at 95°C for 5 minutes. Tris-Glycine SDS-PAGE gels were loaded, and protein extracts were analyzed by electrophoresis in a 10% separating gel. The gels were transferred to polyvinylidene difluoride (PVDF) membranes. After transfer, the membranes were blocked with 5% milk solution and kept in Tris-Buffered Saline containing Tween-20 (TBST) for 1 hour. Membranes were incubated overnight at 4°C with PDE4A Ab and β-Actin Ab. Blots were rinsed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies anti-rabbit for 2 h at room temperature to detect protein bands. Reactive band imaging was performed with an imaging system (ChemiDoc™ MP-BioRad, Hercules, CA, USA) using West-Pico ECL reagents.

Statistical Analysis

Data on cell index were acquired using RTCA integrated software (Santa Clara, CA, USA). ImageJ 1.42 q software (Bethesda, MD, USA) was used to calculate band intensities. SigmaPlot version 15 (Systat Software Inc., San Jose, CA, USA) was used for statistical data analysis. In this study, the significance level was determined at 0.05. Statistically significant values were compared using One-way ANOVA and Dunnett post-hoc test.

Results

RTCA is a system designed to detect cell proliferation and morphological changes without the use of markers. It enables automated, real-time monitoring without requiring direct physiological contact and provides high sensitivity and accuracy imaging. An array of microelectronic cell sensors integrated into electronic plates enables measurement of the electronic impedance of the electrodes and detection and monitoring of changes on the electrodes.

In this research, it was investigated the neuroprotective effects of psoralidin in an Alzheimer's model in which neurotoxicity was induced by MK-801. Psoralidin and MK-801 dose-response curves were obtained by repeated xCELLigence measurements. Cells were treated with the compounds while the index of the cells increased in direct proportion to the number of cells. To obtain growth profiles of HT-22 cells, cells were seeded at 1,250, 2,500, 5,000, 10,000, and 20,000 cells per well. Cell index values increase in proportion to the number of cells. According to the cell index and profile, it was decided that 20,000 cells per well would be the most appropriate number of cells to be planted (Figure 1A). In biological activity studies, MK-801 inhibited cell proliferation in the HT-22 cell line in a concentration-dependent manner. Considering the RTCA results, MK-801 at all concentrations reduced viability in HT-22 cells (Figure 1B). Psoralidin showed a neuroprotective effect at 100 µM, 200 µM and 400 µM concentrations, while 10 µM and 50 µM concentrations reduced the viability of cells treated with 200 µM MK-801 (Figure 1C).

β-Actin and PDE4A expression levels were determined by Western blotting in protein samples acquired after 24 hours of psoralidin and MK-801 treatment. Image bands acquired by Western blotting of PDE4A and β-Actin in HT-22 cells are in Figure 2B. When the PDE4A/β-Actin ratio (Figure 2A) was examined in HT-22 cells, psoralidin 400 µM ($p<0.05$), psoralidin 200 µM ($p<0.05$), (with MK-801 200 µM combined groups) were detected to significantly reduce the PDE4A/β-Actin ratio compared to the control. When the PDE4A/β-Actin ratio (Figure 2A) was examined in HT-22 cells, psoralidin 50 µM ($p<0.01$), psoralidin 10 µM ($p<0.01$), (with MK-801 200 µM combined groups) were detected to significantly increased the PDE4A/β-Actin ratio compared to the control. When the PDE4A/β-Actin ratio (Figure 2A) was examined in HT-22 cells, MK-801 200 µM ($p<0.001$) was detected to significantly increase the PDE4A/β-Actin ratio compared to the control. IC_{50} value of MK-801 was 62.4 µM and the EC_{50} value of psoralidin was 230.4 µM (Table I).

Discussion

This study aimed to determine the potential neuroprotective effects of psoralidin based on PDE4A inhibition and the related mechanism of action by creating a neurodegenerative model with MK-801. Given the limited number and effectiveness of drug molecules currently used to treat neurodegenerative diseases like Alzheimer's, this study aims to assess the long-term effectiveness of its positive findings. The goal is to determine whether these results can further support and enhance existing treatment options. In this study, psoralidin (100, 200, 400 µM) has been shown to have a protective role in MK-801-induced neurotoxicity by the RTCA. In HT-22 cells, the EC_{50} value of psoralidin was calculated to be 230.4 µM, and the IC_{50} value of MK-801 was calculated to be 62.4 µM at 24 hours. It has been determined that psoralidin (200, 400 µM) inhibits PDE4A through the Western blot method.

The EC_{50} value of psoralidin and other findings obtained as a result of this study show that there is a narrow therapeutic window for effective treatment and that it is difficult to reach an effective dose without side effects in the clinical context. Psoralidin should be further investigated for safety and efficacy in terms of a steep drug dose-response relationship, pharmacokinetic and pharmacodynamic measurements followed by therapeutic drug monitoring, and narrow therapeutic index drug properties, including individual variability. A clear understanding of the potential for neuroprotective effects and assessment of the potential for side effects is essential for safe and rational drug administration. Defining an appropriate bioequivalence range for narrow therapeutic index drugs is important due to their efficacy and side effect potential. Small changes in the dosage of narrow therapeutic index drugs may lead to significant changes in pharmacodynamic response, potentially causing subtherapeutic or toxic effects, especially in advanced-stage patients, so their use should be individualized[14]. As with existing narrow therapeutic index drugs, further preclinical and clinical studies are needed to clearly determine

Table I. Potential PDE4A inhibition-mediated neuroprotective effects of psoralidin. EC_{50}/IC_{50} values at 24th h*.

Compound	EC_{50}	IC_{50}
Psoralidin	230.4 µM	–
MK-801	–	62.4 µM

*EC_{50}/IC_{50} values of the molecules were calculated from repeated experiments with RTCA (n=4) and based on the concentration-response curves of the cell index after 24 hours of exposure.

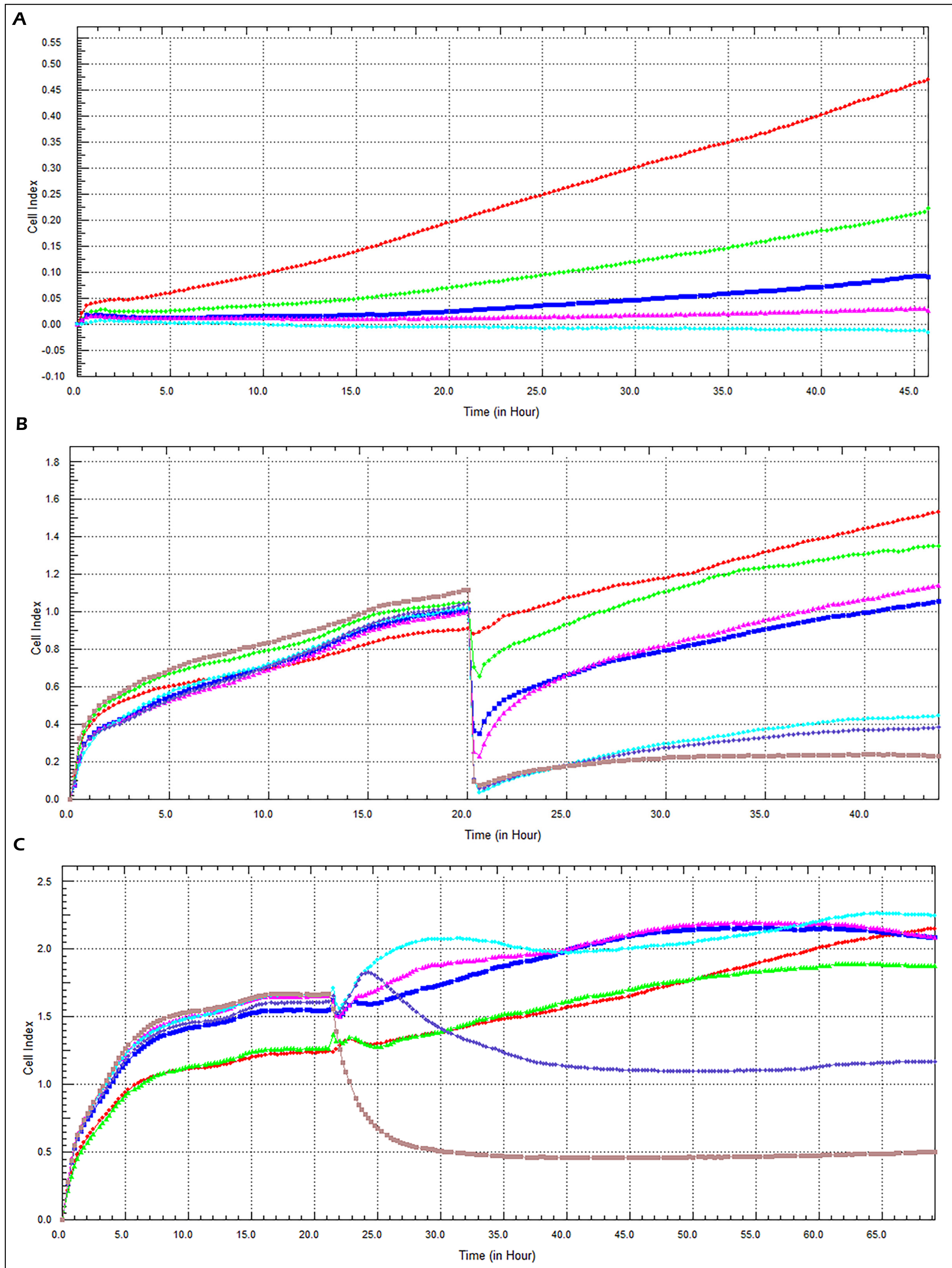

Figure 1. Dynamic monitoring of effects compounds to HT-22 cell proliferation. **A**, The growth profile of HT-22 cells was monitored. **B**, Effects of MK-801 alone. **C**, Effects of MK-801 (200 μM) in combination with psoralidin (10, 50, 100, 200, 400 μM**)** on cell viability.

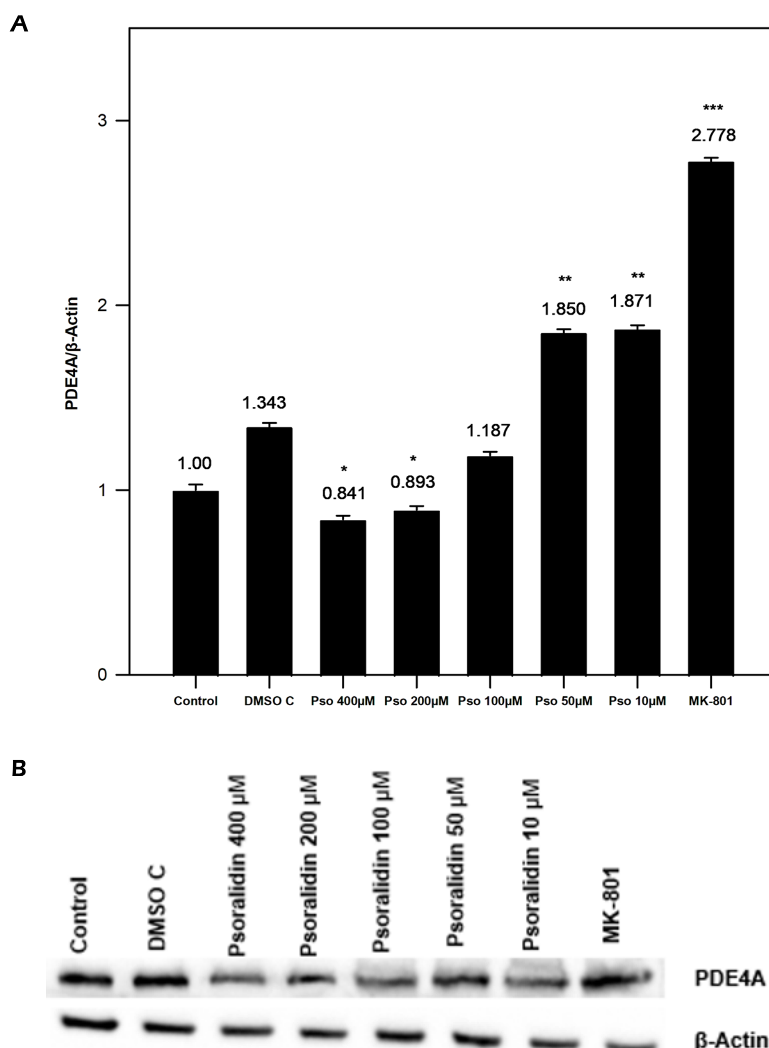

Figure 2. Experimental results were obtained by detection of PDE4A protein expression level *via* Western blotting. **A**, In the bar graph, data represent the relative intensity of PDE4A/β-Actin bands. **B**, PDE4A and β-Actin image bands (24 hours). Results are presented as mean ± SD *p<0.05 *vs.* control. **p<0.01 compared with the control (n=3). C: Control, Pso: Psoralidin.

the degree of efficacy and potential for use. The low oral bioavailability of psoralidin can be increased by various pharmaceutical technology methods. In order to increase bioavailability, lipid-based formulation formation, such as liposomes and nanoemulsions, will be analyzed in further studies.

Active biomolecules are known to promote health and can be used to prevent or treat various diseases, and the detection of active biomolecules is seen as a valuable approach.

Psoralea corylifolia L. (Leguminosae) is a medicinal plant used to treat various ailments[15]. It has cardiovascular system-related effects, as well as anticancer, antibacterial, cytotoxic, and anti-inflammation effects. Literature shows that these plant components have been comprehensively investigated *in vivo* and *in vitro* in different pharmacological and phytochemical studies[16-18]. There are several studies in the literature on the efficacy of psoralidin. In a study, it was stated that Psoralidin isolated from *P. corylifolia* seeds altered the hypothalamus-pituitary-adrenal axis[19]. A similar study involving psoralidin reported that its antidepressant effects were linked to the involvement of monoamine neurotransmitter systems and the hypothalamic-pituitary-adrenal axis[20]. It is also known that when dopamine-producing neurons die, the chemical cannot reach the hippocampus, and memory loss occurs.

In a study in which compounds isolated from *P. corylifolia* were tested for their antioxidant potential, psoralidin was found to be an effective antioxidant with an IC_{50} value of 43.85 mg/L[21]. Nerve cells and brain tissue are more prone to oxidative damage than any other tissue in the body. Moreover, oxidative stress was found to play an important role in the pathogenesis of MK-801-induced neurotoxicity in the cerebellum[22,23].

In an *in vitro* study investigating the effects of *P. corylifolia* and its components on neurons in HT22 and the microglia cell line BV-2, psoralidin was found to significantly suppress LPS-induced NO production in BV-2 cells[24].

Since psoralidin is known to be a compound with low oral bioavailability[9], pharmacokinetic studies are needed to improve its efficacy and distribution.

Conclusions

In this study, a protective effect was observed in neurons with the administration of psoralidin, a natural phenolic coumarin compound, in neurodegeneration caused by MK801-induced accumulation of the excitatory amino acid glutamate. Obtaining data by investigating the mechanisms of action related to PDE4A using the Western blot method may be important for further pharmacological evaluations. Given psoralidin's neuroprotective properties and its pharmacological antioxidant potential, it should be utilized in studies focusing on endogenous defense mechanisms and specific pathways of oxidative damage for further evaluation. Long-term studies will be significant in the evaluation of its efficacy.

Conflict of Interest
The author declared no conflict of interest.

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Informed Consent
Not applicable.

Ethics Approval
Institutional ethical approval was not required for this study.

Availability of Data and Materials
The data presented in this article are available from the corresponding author upon reasonable request.

Authors' Contributions
E.U. planned the study. E.U. conducted experiments. E.U. did the analysis. E.U. wrote the manuscript.

AI Disclosure
The author declares that no artificial intelligence or assisted technologies were used in the production of this study, including the creation of any figures or the writing of the article.

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