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Anti-inflammatory effects of Iturin A on the 6-OHDA-induced SH-SY5Y cell model of Parkinson's Disease

Iturin A'nın 6-OHDA ile indüklenmiş SH-SY5Y parkinson hastalığı hücre modeli üzerindeki anti-inflamatuar etkileri

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ABSTRACT

Aim: Biosurfactants produced by bacteria are particularly important due to their antibacterial, antifungal, and antiviral properties, which offer therapeutic and biomedical potential. Nevertheless, no research has examined the impact of Bacillus-derived biosurfactant Iturin A on Parkinson's disease. This study examined the neuroprotection through cytokine modulation of Iturin A to formulate a novel therapeutic approach for Parkinson's disease.

Materials and Methods: The effect of Iturin A on cell viability in SH-SY5Y cells was evaluated by the [3-(4,5-Dimethyl thiazole-2) 2,5-diphenyltetrazolium bromide] method. As a result of this experiment, the non-toxic dose was determined by cell viability (%). To evaluate the neuroprotective effect of Iturin A, SH-SY5Y cells were treated with a non-toxic dose of Iturin A and then induced with 6-OHDA. After these treatments, cytokines (interleukin 6, transforming growth factor beta) and chemokine (interleukin 8) in SH-SY5Y cells were evaluated by ELISA kit. GraphPad Prism 9 was used to analyze all data.

Results: Using one-way ANOVA and Dunn's post hoc test, normally distributed data was tested for multiple comparisons**.** Treatment with Iturin A for 48 hours showed a neuroprotective effect on 6-hydroxy dopamine-induced SH-SY5Y cells by statistically significantly decreasing the expression levels of interleukin 6 ($P<0.001$), transforming growth factor beta ($P<0.001$), and interleukin 8 ($P<0.0001$).

Conclusion: Iturin A might offer new approaches to treating Parkinson's patients, and additional *in vivo* studies are necessary to confirm the *in vitro* results.

Keywords: Parkinson's Disease, Biosurfactants, Neuroinflammation, Cytokines

ÖZ

Amaç: *Bakteriler tarafından üretilen biyosürfaktanlar, terapötik ve biyomedikal potansiyel sunan antibakteriyel, antifungal ve antiviral özellikleri nedeniyle özellikle önemlidir. Bununla birlikte, hiçbir araştırma Bacillus türevi biyosürfaktan İturin A'nın Parkinson hastalığı üzerindeki etkisini incelememiştir. Bu çalışma, Parkinson hastalığı için yeni bir terapötik yaklaşım formüle etmek amacıyla İturin A'nın sitokin aracılı nöroprotektif etkisini incelemiştir.*

Gereç ve Yöntem: *İturin A'nın SH-SY5Y hücrelerindeki hücre canlılığı üzerindeki etkisi MTT [3-(4,5-Dimetil tiyazol-2) 2,5-difeniltetrazolium bromür] yöntemi ile değerlendirilmiştir. MTT deneyinin sonucunda, toksik olmayan doz hücre canlılığı (%) ile belirlendi. İturin A'nın nöroprotektif etkisini değerlendirmek için, SH-SY5Y hücreleri toksik olmayan bir doz İturin A ile muamele edildi ve ardından 6-OHDA ile indüklendi. Bu muamelelerden sonra, SH-SY5Y hücrelerindeki sitokinler (IL-6, TGF-β) ve kemokin (IL-8) ELISA kiti ile değerlendirildi. Tüm verileri analiz etmek için GraphPad Prism 9 kullanıldı.*

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Bulgular: *İturin A ile 48 saatlik tedavi, sitokinlerin (IL-6, TGF-β) ve kemokin (IL-8) ifade seviyelerini değiştirerek 6-OHDA ile indüklenen SH-SY5Y hücrelerinde nöroprotektif bir etki gösterdi (p<0,05 - p<0,0001).*

Sonuç: *İturin A, Parkinson hastalarının tedavisinde yeni yaklaşımlar sunabilir ve in vitro sonuçları doğrulamak için ek in vivo çalışmalara ihtiyaç vardır.*

Anahtar Sözcükler: *Sitokinler, İnflamasyon, İturin A, Nöroprotektif, SH-SY5Y*

INTRODUCTION

Parkinson's disease (PD), characterized by motor and non-motor features, is a progressive neurological disease (1). Neuroinflammation mediated by microglia is a common feature in PD (2). Although the exact etiology of PD remains unknown, the loss of dopaminergic neurons in the substantia nigra (SN) is thought to be the underlying pathophysiology behind the gross motor symptoms of the disease. Recent studies have shown that PD is associated with a strong inflammatory response characterized by activation of microglia in the brain due to increased cytokines (3). PD leads to a significant increase in cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, IL-6, transforming growth factor (TFG)-α, TGF-β1 and TGF-β2 (4)**.** Although the factors underlying the neurodegenerative cascade, such as aging, environmental and genetic factors, are known the intricate processes responsible for the progression of neurodegeneration are still not fully understood (5). TGF-β, IL-6, and IL-8 are among the cytokines that may be used as PD biomarkers. These cytokines contribute significantly to neuroinflammatory characteristics. Their profiles could be useful for neurodegenerative disease prognosis, early detection, and treatment result documentation (6–10).

Excessive production of reactive oxygen species (ROS) and inadequate antioxidant defenses lead to mitochondrial damage and dysfunction, and ultimately to the pathogenesis of PD (11). Due to the high mortality and morbidity associated with this disease, the development of diagnostics and drugs to modify the disease is an urgent medical need for PD (12). Therefore, it is important to understand the pathophysiological mechanisms underlying PD (13). Biosurfactants are surface-active compounds produced by some biological agents such as yeast and bacteria (14). Scientific studies show that they are safer than chemical surfactants, making them a preferred alternative in terms of the environment (14). The anti-inflammatory, antioxidant and antibacterial potential of biosurfactants has been demonstrated in numerous studies (15–17). Iturin A is a type of lipopeptide produced by *Bacillus* spp. (18). It has multiple activities inhibiting fungi, bacteria and viruses (19). Iturin A biosurfactant with a fatty acid chain and a cyclic peptide isolated from the fermentation of *Bacillus subtilis* exhibits a variety of biological activities and is of interest due to its anti-cancer properties (20,21). Iturin A also exhibits strong anti-inflammatory property on cells (22). Although the mechanisms that affect these anti-inflammatory processes are not fully elucidated, it is known to affect specific inflammatory pathways such as Nuclear Factor kappa B NF-κB (23). This study examined the neurotherapeutic effect of Iturin A biosurfactant via some inflammatory cytokines in the 6-hydroxy dopamine (6-OHDA)-induced *in vitro* PD model.

MATERIALS and METHODS

Cell Culture

Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12) (Gibco, Thermo Fisher Scientific, UK), supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit-Haemek, Israel) and 2% penicillin-streptomycin (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) were used to culture human neuroblastoma cell lines SH-SY5Y (CRL-2266) purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The culture grew at 37 °C with 5% CO_2.

Cell Proliferation and Cytotoxicity Assays

After the cultures of SH-SY5Y cells were 80–90% confluent, the cells were detached with 0.05% trypsin-EDTA (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) for two minutes and then centrifuged for five minutes at 1200 rpm. Trypan blue staining was used to determine the total number of cells using an automatic cell counter (Luna II, Logos Biosystems, Gyeonggi-do, South Korea). To perform the 3-(4,5-dimethyl-2-

thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sigma, Missouri, ABD), 96-well plates were seeded with 5×10^3/well SH-SY5Y cells, which were then incubated for 24 hours. Subsequently, Iturin A (No: I1774, Sigma-Aldrich, Saint Louis, USA) was added to the SH-SY5Y cells at six different concentrations (30, 40, 50, 60, 70, and 80 µM) for a 48-hour treatment. As a positive control, SH-SY5Y cells were treated with L-DOPA at doses ranging from 0.05 to 1.3 µM for 48 hours. After a 48-hour incubation period, 10 µl of MTT solution (5 mg/ml in DPBS) was added to each well and the cells were incubated for a further three hours. After discarding the medium, 100 µL of DMSO was given into each well. A microplate reader (TECAN-sunrise, Männedorf, Switzerland) was used to determine the viability of the cells at 570 nm. Each experiment was done three times. Using a range of Iturin A concentrations, the 50% inhibitory concentration (IC_{50}) value (48.709 µM) was calculated using GraphPad Prism version 9 software. Non-linear regressions of log (inhibitor) versus response with four parameters were selected for IC_{50} estimation. Only 0% and 100% could be the low- and high-end values, respectively.

Establishment of an *in vitro* Parkinson's Disease Model

Neurotoxicity was induced by 6-OHDA (Sigma, H4381), which can disrupt cellular signaling pathways related to mitochondrial damage and oxidative stress, in SH-SY5Y cells. The 6-OHDA stock concentration was freshly prepared and was 10 mM in 1 mL of solvent (0.01% (w/v) L-ascorbic acid (Sigma, A4403). Then, this stock was diluted in the medium to 200 µM, 300 µM, 400 µM and 500 µM concentrations (2X) to be used on the cells, and the appropriate concentration was determined in SH-SY5Y cells by MTT method. After the SH-SY5Y cells adhered to the 6-well plates, they were incubated for 48 hours to evaluate the protective effect of Iturin A. Subsequently, the cells were incubated with 6-OHDA at 250 µM concentration for 24 hours to induce neurodegeneration after 48 hours. The group treated with Iturin A was compared to the 6-OHDA group and negative control by not treating them.

ELISA Assay

For the ELISA assay, SH-SY5Y cells (1×10^6/mL) were seeded in 6-well plates and incubated for 24 hours. Iturin A was added at a concentration of 30 µM, and the cells were incubated with 6-OHDA for 24 hours to induce neurodegeneration after 48 hours. At the end of the incubation, the supernatant of the cells was collected. Direct competitive chemiluminescence ELISA kits (Bioassay Technology Laboratory, Shanghai, China) were used to measure the levels of IL-6, IL-8, and TGF-β in the supernatant (extracellular) according to the manufacturer's instructions. The cell culture supernatant was collected in sterile Eppendorf tubes after centrifugation at 2000-3000 rpm for about 20 minutes to determine cytokines released extracellularly. 50 µL of each diluted cell pellet (1:40 in 1×PBS, pH 7.2-7.4) was lysed by multiple freeze-thaw cycles to remove internal components to determine the intracellular cytokines and chemokines. After centrifugation at 2000 rpm for 20 minutes, the supernatants were collected in sterile Eppendorf tubes. A microplate reader (TECAN-sunrise, Männedorf, Switzerland) was used to determine the absorbance of the samples at 450 nm.

Statistical Analysis

GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA) was used to analyze all of the data. In paired comparisons, the Mann-Whitney U test was used to evaluate non-normal data while the student's t-test was used to analyze regularly distributed data. Using one-way ANOVA and Dunn's post hoc test, normally distributed data was tested for multiple comparisons. Tukey's post hoc adjustment was applied to the Kruskal Wallis test when analyzing non-normally distributed data. $p< 0.05$ value was considered statistically significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.001$).

RESULTS

Evaluation of the Cytotoxicity of Iturin A in the SH-SY5Y Cells

First, SH-SY5Y cells were treated with Iturin A at different concentrations (30, 40, 50, 60, 70, and 80 µM) for 48 hours. Cell viability decreased with increasing concentrations (Figure-1). The cell viability of SH-SY5Y cells remained almost unchanged when treated with Iturin A up to a concentration of 30 µM, indicating the non-toxicity of Iturin A. Cell viability was 85.2% at a concentration of 30 µM. The subsequent experiments employed Iturin A at a concentration of 30 µM because it showed cytotoxic effects at higher concentrations than 30 µM and the IC_{50} value was determined as 48.709 µM by calculating the effect of increasing concentrations of Iturin A

on cell viability (%). For subsequent experiments, the non-toxic dose of Iturin A, 30 µM, was used.

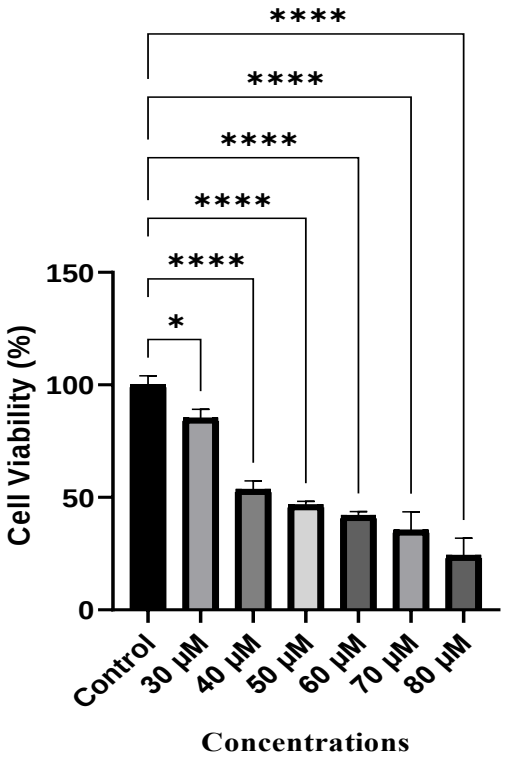

Figure-1. Iturin A decreases the viability of SH-SY5Y cells due to increasing concentration. SH-SY5Y cells were treated with six different Iturin A concentrations for 48 hours. The IC_{50} value was calculated as 48.709 µM. The significant difference compared to the control group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The Effect of Iturin A on 6-OHDA-Induced Cytotoxicity in SH-SY5Y Cells

SH-SY5Y human neuroblastoma cells are commonly used for 6-OHDA-induced *in vitro* PD model (24). In agreement with previous studies, 6-OHDA showed a cytotoxic effect on the cells. After an application of a 250 µM concentration, cell viability was expressed as a percentage and an IC_{50} was calculated. Iturin A at a concentration of 30 µM dramatically improved cell viability (104.27%) similar to that in the L-DOPA group (125.08%), compared to the 6-OHDA-induced neurotoxicity group (Figure 2). Since the lowest concentration of Iturin A provided the greatest benefit, a concentration of 30 µM Iturin A was used for further experiments.

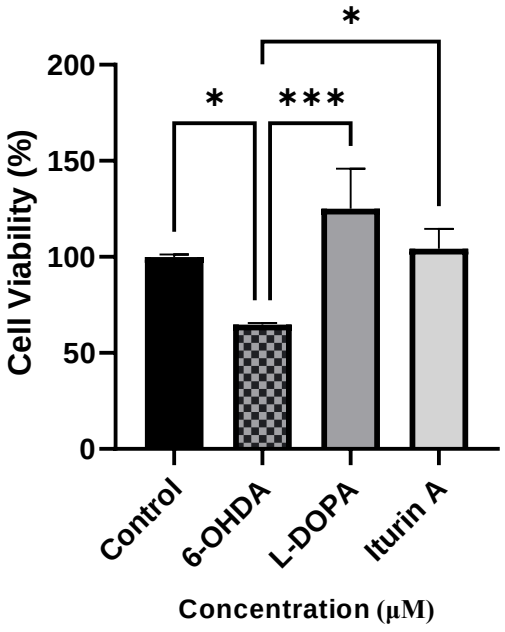

Figure-2. Iturin A reduces cytotoxicity in 6-OHDA-induced SH-SY5Y cells. Significant difference compared to control and 6-OHDA-induced neurotoxicity groups, *p<0.05, ***p<0.001.

Iturin A Reduces the Inflammatory Response Induced by 6-OHDA in SH-SY5Y Cells

TGF-β, IL-6, and IL-8 levels increased in the 6-OHDA-induced SH-SY5Y cells compared to the control group (Figure-3). The therapy with Iturin A in 6-OHDA-induced SH-SY5Y cells dramatically decreased the production of TGF-β, IL-6, and IL-8 compared to the 6-OHDA-induced neurotoxicity group (Figure-3).

While the IL-6 level increased statistically significantly in 6-OHDA-induced SH-SY5Y cells compared to the control group (P<0.001), it significantly decreased in these cells treated with Iturin A compared to 6-OHDA-induced SH-SY5Y cells (P<0.001). There was a decrease in IL-6 level in L-DOPA-treated cells (positive control), but it was not statistically significant (P>0.05) (

Figure 3.A). IL-8 level significantly increased in 6-OHDA-induced SH-SY5Y cells compared to the control group (P<0.01) and significantly decreased in Iturin A-treated cells compared to 6-OHDA-induced SH-SY5Y cells (P<0.0001). IL-8 levels significantly decreased in L-DOPA-treated cells (positive control) (P<0.0001) (Figure 3.B). Similarly, TGF-β level significantly increased in 6-

OHDA-induced SH-SY5Y cells compared to the control group (P<0.05) and significantly decreased in Iturin A-treated cells compared to the 6-OHDA-induced cells (P<0.01). TGF-β level significantly decreased in L-DOPA-treated cells (positive control) (P<0.01) (Figure-3.C)

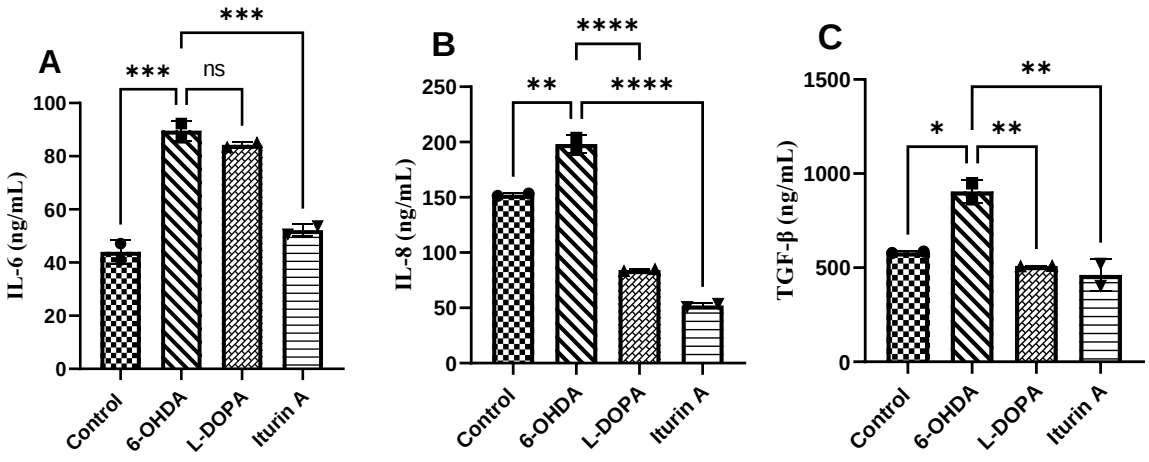

Figure-3. Iturin A reduces inflammation in 6-OHDA-induced SH-SY5Y cells. IL-6 (A), IL-8 (B), and TGF-β (C) protein expressions. Significant difference compared to control and 6-OHDA-induced neurotoxicity groups, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

DISCUSSION

The prevalence and incidence of PD are increasing worldwide as the population ages, and it is estimated that there will be approximately 30 million PD patients worldwide by 2030 (25). The disease is a major public health concern because it becomes more common with increasing age (26). There is currently no cure for PD, but clinical trials are investigating potential disease-modifying approaches (27,28).

Biosurfactants, particularly Iturin A, are attracting attention due to their anti-inflammatory effects (22). Although the importance of biosurfactants in developing strategies to prevent and treat the disease is well known, studies in this area are quite limited. Although there are no studies in the literature on the effect of Iturin A in the treatment of PD, it cannot be ignored due to its effects on neurodegeneration. (29–31)**.** The cytotoxic, anti-/pro-inflammatory, and anti-tumor properties of the biosurfactant Iturin A were investigated in this work using ELISA and MTT assays on an *in vitro* PD cell model.

The source of Iturin A used in our study is a Bacillus from the soil. As far as we know, there is no information in the literature about the anti-tumor properties of Iturin A obtained from the same source in SH-SY5Y cells. In this context, our study is of particular importance. In this study, we calculated the IC_{50} value for Iturin A in SH-SY5Y cells as 48.709 µM for 48 hours. Moreover, Iturin A decreased the viability of SH-SY5Y human neuroblastoma cells to below 50% and lowered neurotoxicity.

The 6-OHDA-induced *in vitro* PD model is a useful model to investigate the molecular basis of cytotoxicity, to study the cellular processes activated by neuroinflammation and neuronal death, and to understand new treatment mechanisms, as it reproduces the cellular processes described in PD (32). The biology of PD and the efficacy of potential neuroprotective drugs are being investigated using SH-SY5Y cells as an experimental model (33).

There are a large number of activated microglial cells in the substantia nigra striatum of PD patients, which produce various cytokines (e.g. TNF-α, IL-6 and IL-1β, etc.) and then induce an inflammatory response and promote neuronal lesions (34,35). Therefore, reducing microglial inflammation is a useful approach for the treatment of PD (36) Previous studies have shown that levels of various cytokines such as TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, epidermal growth factor (EGF), TGF-α, TGF-β are elevated in PD (10,37–39).

Interleukins are released by activated microglia and leukocytes. IL-6 is a multifunctional cytokine secreted by neurons and glial cells and IL-6 plays an important role in neuronal development and differentiation (38). They possess a number of receptors, one of which is IL-6, which promotes neuronal cells survival at normal concentrations but can cause neurodegeneration when overactivated (40). In our study, 6-OHDA-induced SH-SY5Y cells, which we created to mimic the PD model, had elevated IL-6 levels in line with neurodegeneration. Iturin A treatment reduced inflammation in 6-OHDA-induced SH-SY5Y cells by lowering IL-6 levels.

Interleukin-8 (IL-8/CXCL8), a major CXC chemokine, exerts profound effects on cell-cell activation and brain function, suggesting possible involvement in neuroinflammation (41). IL-8 is secreted by a variety of cell types and is released when an inflammatory trigger occurs (42,43). IL-8 levels are particularly high in Parkinson's patients (10). In our study, 6-OHDA-induced SH-SY5Y cells had higher IL-8 levels than the control group. Similar to the L-DOPA treatment group (positive control), treatment with Iturin A reduced the levels of IL-8 in these neurodegenerated cells.

PD is associated with higher TGF-β levels, suggesting a possible modulatory involvement in neurodegeneration (44,45). Elevated TGF-β levels are even protective against neurotoxicity (46). In our study, we found that the 6-OHDA-induced neurotoxicity group had higher TGF-β levels. Similar to the positive control L-DOPA group, treatment with Iturin A dramatically decreased inflammation in these neurotoxic cells by lowering TGF-β levels.

In addition, when all these findings are evaluated together, these cytokines/chemokines seem to work synergistically and thus contribute to neurodegeneration and neuroprotection by supporting each other.

CONCLUSION

The expression of cytokines, which increases and/or decreases during biosurfactant treatment, could contribute to the development of PD. In the present study, the biosurfactant Iturin A was shown to decrease the increased levels of IL-6, IL-8 and TGF-β in 6-OHDA-induced SH-SY5Y cells, an *in vitro* PD model. High levels of these cytokines are known to be particularly common in PD. In conclusion, the Iturin A treatment has a protective effect against the toxicity of 6-OHDA in SH-SY5Y cells. However, these findings are preliminary and were studied *in vitro* based on a single cell line. In this context, it is supported *in vivo* with additional cytokines and chemokines to better understand its efficacy for clinical translation.

Credit authorship contribution statement

Pinar Altin Celik: Conceptualization, Investigation, Data curation, Methodology, Validation, Writing-original draft, Writing – review & editing. **Muazzez Derya Andeden:** Methodology, Investigation, Writing – review & editing. **Hamiyet Donmez Altuntas:** Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Conflicts of interest: Authors declared no conflict of interest.

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