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Neuroprotective effects of a Wnt antagonist in quinolinic acid-induced excitotoxicity in N18D3 cells

Jinsu Hwang[1,$], Jong-Seong Park[1,$], Mahesh Ramalingam[1], Byeong C. Kim[2], Han-Seong Jeong[1,*], Sujeong Jang[1,#]

[1] Department of Physiology, Chonnam National University Medical School, Jellanamdo 58128, Republic of Korea
[2] Department of Neurology, Chonnam National University Medical School, Gwangju 61469, Republic of Korea

[$]Equally contributed, *Co-corresponding author

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ABSTRACT

Excessive stimulation of the quinolinic acid induces neuronal cell death and is implicated in developing several neurodegenerative diseases. This study investigated whether a Wnt5a antagonist plays a neuroprotective role by regulating the Wnt pathway, activating cellular signaling mechanisms, including MAP kinase and ERK, and acting on the antiapoptotic and the proapoptotic genes in N18D3 neural cells. The cells were pretreated with a Wnt5a antagonist Box5, for one hour and then exposed to quinolinic acid (QUIN), an NMDA receptor agonist for 24 hours. An MTT assay and DAPI staining were used to evaluate cell viability and apoptosis, respectively, demonstrating that Box5 protected the cells from apoptotic death. In addition, a gene expression analysis revealed that Box5 prevented the QUIN-induced expression of the pro-apoptotic genes, BAD and BAX, and increased that of the anti-apoptotic genes, Bcl-xL, BCL2, and BCLW. Further examination of potential cell signaling candidates involved in this neuroprotective effect showed that the immunoreactivity of ERK was significantly increased in the cells treated with Box5. These results suggest that the neuroprotective mechanism of Box5 against QUIN-induced excitotoxic cell death involves the regulation of ERK and modulation of cell survival and death genes through decreasing the Wnt pathway, specifically Wnt5a.

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Introduction

In the nervous system, neurodegenerative diseases represent a diverse spectrum of neuronal disorders with progressive dysfunctions. These diseases are identified by the presence of disease-specific protein aggregations and misfolding in specific brain regions (1). The prevalence and incidence of neurological disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, and amyotrophic lateral sclerosis, increase with age and peak well before the maximum lifespan (1). PD, the second most diagnosed neurodegenerative disease, is increased in the older population, affecting almost 1% of the population over age 60 (2). Typical neuropathological hallmarks of PD include the selective loss of dopaminergic cell bodies in the substantia nigra pars compacta with consequent depletion of striatal dopamine, the deposition of cytoplasmic fibrillary inclusions containing ubiquitin and α-synuclein, and astroglial activation (2). To date, there are no effective treatments that can stop or reverse the neurodegeneration process in PD, and current treatments rely on dopaminergic drugs, including L-DOPA and dopaminergic agonists. The wingless-type (Wnt) signaling pathway, known for its crucial role in age-related disease pathogenesis, is one key regulator of dopaminergic neurogenesis promoting tissue repair and regeneration of cells (2). Excessive Wnt signaling is also detrimental to dopaminergic neuron production, adding to the general notion that morphogen signaling must be tightly regulated (2).

Wnt proteins are a family of glycoproteins that comprise two major pathways; the canonical or β-catenin-dependent signaling pathway, which is activated by Wnt1, Wnt2, Wnt3, Wnt3a, and Wnt7a, and the non-canonical or β-catenin-independent signaling pathway, which is activated by Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b, and Wnt11. Wnt signaling is crucial for organizing complex cellular behaviors during development, regeneration, and cellular homeostasis, where it mediates cell proliferation, polarity, differentiation, motility, and activity (3). Depending on the type of Wnt interactions and downstream involvement or absence of β-catenin, Wnt signaling pathways can be grouped into two distinct downstream classes, namely, the β-catenin-dependent canonical pathway and β-catenin-independent non-canonical pathways, including the planar cell polarity and the Wnt/Ca2+ pathways (3). The Wnt/β-catenin pathway has been most extensively studied in neurodegeneration (1, 4). Depleted levels of β-catenin have been correlated with synaptic loss and amyloid beta (Aβ) deposition. They have been identified as key factors before the onset of neuronal death in many neurodegenerative diseases, including AD (1). Several studies on β-catenin in neuronal cells have reported that neural depolarization

* Corresponding author. Email: sujeongjang@jnu.ac.kr

induces β-catenin redistribution from dendritic shafts into spines, interacting with cadherin to influence synaptic size and strength (1).

Reduced Wnt signaling is detrimental to injury repair (5, 6). The most prominent pathway-activating protein in Wnt signaling research, glycogen synthase kinase 3 beat (GSK-3β), has several small molecules associated with it as well (5). Because of the implication of Wnt signaling in various cancers, inhibiting Wnt has helped determine how Wnt signaling is regulated and has uncovered more clinically useful insights (5). Studies focused on Wnt signaling and mitochondria have found that β-catenin-dependent Wnt signaling, via the Wnt3a ligand, prevents the permeabilization of mitochondrial membranes induced by Aβ oligomers. These studies also found that β-catenin-independent Wnt signaling, through the Wnt5a ligand, protects mitochondria from mitochondrial fission-fusion alterations that occur in AD and modulates B-cell lymphoma 2 (Bcl-2) increases (1, 7). Wnt signaling is employed to orchestrate fundamental developmental processes in early development and later during the growth and maintenance of various tissues (4, 8). Mutations in Wnt genes or Wnt pathway components lead to specific developmental defects. Various human diseases, including many different types of cancer and neurodegenerative diseases (AD, PD, and brain injury), are caused by abnormal Wnt signaling (5, 8). Wnt/β-catenin signaling is a vital pathway for dopaminergic neurogenesis and an essential signaling system during embryonic development and aging, the most critical risk factor for PD (2, 4).

Box5, a Wnt antagonist, is a Wnt5a-derived hexapeptide that is well known to antagonize Wnt5a-mediated cellular activities including, migration, invasion, calcium response, and MARCKS phosphorylation, effectively (9). In addition, Wnt5a is conserved in multiple species, including humans, mice, and rats, and Box5 has been shown to antagonize its effect via direct frizzled class receptor 5 (FZD5) binding (10). It is well known that the Wnt5a-mediated c-Jun N-terminal kinase 3 (JNK3) signaling pathway is correlated with neuronal death in neurodegenerative diseases such as brain injury. Wei's group found that Box5 and mimic peptides play protective roles in neuronal death both in vivo and in vitro in global and focal cerebral I/R rat models and oxygen-glucose deprivation (OGD) neural cells, respectively. This study concluded that the Wnt5a-JNK3 signaling pathway might be a new target for stroke therapy (11). Box5 also reduced stroke size, suggesting that decreasing FZD5 and enhancing p65/NFκB downstream endocytosis reduced Wnt5a expression and neuronal cell death (9). Given the protective effect of Box5 in neuronal death, we investigated whether Box5 protects neuronal cells from quinolinic acid (QUIN)-induced excitotoxicity by evaluating the mRNA expression levels of apoptosis-related genes.

Materials and Methods

Cell lines and reagents

Box5 (t-boc-Met-Asp-Gly-Cys-Glu-Leu hexapeptide; Screen-Well® Wnt pathway library, Enzo Life Sciences, Inc., Farmingdale, NY, USA) was prepared as a stock solution in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO, USA). The final DMSO concentration was < 0.1% in all the experiments. Quinolinic acid (QUIN; Sigma Chemical Co.) was prepared as a stock solution in distilled ultra-pure water.

N18D3 cells, a hybrid neuronal cell line obtained by the fusion of dorsal root ganglions isolated from a 4-week-old Balb/C mouse with mouse neuroblastoma N18TG2 cells, were grown as adherent cultures in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY, USA), and 0.2% amphotericin B (Gibco BRL) in a 37°C humidified incubator with 5% CO2 (12).

Experimental methods

Assessment of cell viability

To assay cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) was prepared following our previously published methods (12). In brief, cells were plated at a density of 1 x 104 cells/mL in 96-well plates (Nalge Nunc International, Rochester, NY, USA) and then incubated with Box5 (10 μM) for 24 hours before the addition of 30 mM QUIN. Twenty-four hours after QUIN exposure, 20 μL MTT (5 mg/mL) were added to each well, and the cells were incubated for 4 hours at 37°C in the dark. The formazan precipitate was dissolved in 100 μL DMSO and the optical density (OD) at 570 nm was evaluated on an ELISA plate reader (Bio-Tek μQuant, ReTiSoft Inc., Ontario, Canada). All results were calibrated to the OD measured in the same conditioned well without cell culture. The MTT assay was repeated four times independently and values from each treatment were calculated as a percentage relative to the untreated control (defined as 100% survival).

To evaluate the nuclear morphology after treatment with Box5 and QUIN, 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) staining was conducted according to our previous study's methodology (12), and then the cell nuclei were observed using a fluorescence microscope (ImageXpress Nano, Molecular Devices, CA, USA). The number of apoptotic cells, defined as DNA fragmentation, nuclear condensation, and segmentation, were identified in at least 400 cells.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

To evaluate the expression of related genes, the cells were cultured and prepared following the experimental procedure and our previous methods (13, 14). Total RNA was isolated following the TriZol-based protocol according to the manufacturer's instructions, and cDNA was synthesized using a reverse-transcribed system (Takara Bio Inc., Shiga, Japan). The cDNA was amplified by 35 cycles of 94°C for 2 minutes, appropriate annealing temperatures for each primer for 1 minute, and 72°C for 1 minute, and then the RT-PCR products were observed. Quantitative analysis was performed with the Image J software. The mRNA expression was normalized based on the level of ubiquitously expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All experiments were repeated five times independently.

Western blot assay

The cells were cultured and prepared for a western blot assay following our previous methods (15-18). Wnt

family proteins and the phosphorylated and total forms of the enzymes, extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38, and JNK, were observed via western blot analyses, as described previously. Briefly, the cells were lysed in a lysis buffer including protease inhibitors and phosphatase inhibitors and the protein concentration was determined with the Quant-iTTM assay kit (Molecular Probes, Eugene, OR, USA). The protein (15 mg/mL) was loaded into 8-12% sodium dodecyl sulfate-polyacrylamide electrophoresis gel and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were incubated with specific antibodies for Wnt1, Wnt2, Wnt3, Wnt4, Wnt5a, Wnt7a/b, Wnt10a, phosphatidylinositol-3-kinase (PI3K), p-PI3K, protein kinase C (PKC), p-PKC, ERK1/2, p-ERK1/2, JNK, p-JNK, p38, p-p38, GSK3β, p-GSK3β, or β-actin. Antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), and the dilution factor was 1:2000. The signals were detected by enhanced chemiluminescence reagents, and images were detected on ImageQuant LAS4000 (GE Healthcare, Piscataway, NJ, USA). The density was quantified using the Image J software. The protein expression was normalized based on the level of β-actin. All experiments were repeated at least three times independently.

Experimental data processing

Protein and mRNA levels were quantified by measuring the optical density of each band using computer-assisted densitometry (NIH Image analysis program, version 1.61). A one-way analysis of variance (ANOVA) test (Bonferroni post-hoc comparison) was used to analyze the differences between groups, with $p < 0.05$ being considered significant. All values were expressed as the mean ± standard error of the mean (SEM).

Results

Box5 protects against QUIN-induced cell death

To evaluate the neuroprotective effects of Box5 pretreatment on excitotoxicity, we performed an MTT assay to evaluate cell viability and DAPI staining to detect DNA fragmentation and condensation. The viability of N18D3 neuronal cells was examined after Box5 and/or QUIN treatment for 24 hours. When cultured cells were exposed to 30 mM QUIN, the cell survival rate was 43.5 ± 2.0055% (**$p < 0.01$). However, when the neuronal cells were co-incubated with Box5 before QUIN treatment, the percentage of survival cells increased significantly to 52.4 ± 1.9425 % (##$p < 0.05$) at 10 µM (Figure. 1A). In parallel, fewer cells with nuclear condensation and DNA fragmentation, characteristic features of apoptosis, were observed in Box5-pretreated cells. In contrast. those features were highly increased in QUIN-only treated cells via DAPI staining (Figure. 1B). Untreated cells and those treated with Box5 alone displayed a round or oval-shaped nucleus. In addition, quantification data exhibited a decreased number of apoptotic cells from 13.4003 ± 1.4845 % (**$p < 0.01$) in QUIN-treated cells to 5.935 ± 0.4745 % in cells co-incubated with Box5 before QUIN treatment (Figure. 1C). These results support the notion that Box5 protects against QUIN-induced neurotoxicity in N18D3 cells.

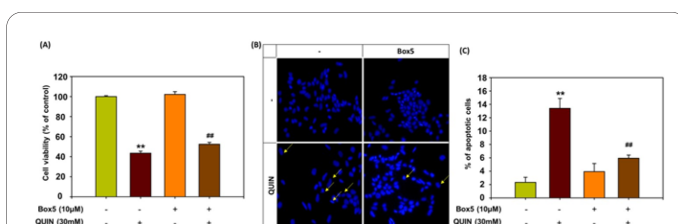

Figure 1. Protective effect of Box5 against QUIN-induced excitotoxicity. (A) We treated N18D3 cells with Box5 (10 µM) prior to exposing them to QUIN (30 mM) for 24 hours. Pretreatment with Box5 improved cell viability. (B) DAPI staining showed condensation and fragmentation, which are characterized features of apoptosis. The treatment of QUIN caused apoptotic-like nuclear changes, whereas Box5 pretreatment prevented such changes from occurring. The scale bar indicates 20 µm. (C) The apoptotic cell population was reduced by Box5 pretreatment. **$p < 0.01$ compared with the control; ##$p < 0.05$ compared with the QUIN-treated cells.

Analysis of Wnt family genes in N18D3

To identify Wnt family genes at the molecular level, we performed RT-PCR analysis of both canonical and non-canonical Wnts (WNT1, WNT3, WNT4, WNT5A, WNT7B, WNT10B). The cells were incubated with Box5 prior to QUIN for six hours. Total RNA was then isolated, and cDNA was prepared for RT-PCR analysis. The level of WNT5A was increased with QUIN and Box5 co-treatment, while it was decreased with Box5 treatment alone. In addition, the expression of WNT1 and WNT10B decreased significantly with QUIN and Box5 co-treatment (Figure 2). WNT4, one of the non-canonical Wnts, was not changed after treatment of QUIN and/or Box5. In non-canonical signaling, we found any expression of WNT7B, and there was no change in WNT10B expression. However, the level of WNT3 was increased with Box5 treatment and recovered with QUIN.

Box5 inhibits the QUIN-induced expression of apoptotic genes

To validate the neuroprotective action of Box5, the mRNA expression of apoptosis-related genes was performed using RT-PCR. We previously demonstrated that QUIN regulates mRNA expression by inducing pro-apoptotic genes and reducing anti-apoptotic genes (12). Here, we studied the effect of Box5 pretreatment on the expression of apoptosis-related genes induced by QUIN in N18D3 cells. We found that QUIN stimulated the expression of pro-apoptotic genes, such as caspase 3 (CASP3) and CASP9. At the same time, pretreatment with Box5

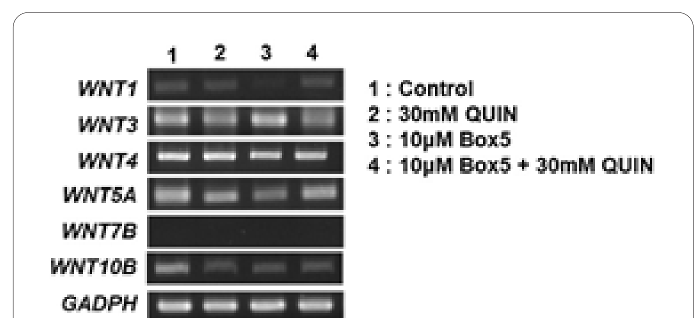

Figure 2. RT-PCR analysis of Wnt family genes. We measured the expression of Wnt genes by RT-PCR. GAPDH was used as a control. The RT-PCR assay was repeated five times independently in different cells and representative data are shown.

(10 μM) inhibited these gene expression elevations (Figure. 3). In contrast, QUIN inhibited the expression of the anti-apoptotic gene, such as B-cell lymphoma extra-large (Bcl-xL) and Bcl-2-like protein 2 (BCLW), whereas Box5 pretreatment prevented this inhibition. The level of BCL2 was not changed with QUIN or Box5. Interestingly, the anti-apoptotic gene expression increased in the cells treated with Box5 alone as compared to the controls. These results imply that Box5 has protective effects on various apoptosis-related processes.

Wnt and ERK signaling mediated the protective effects of Box5 in excitotoxicity

To investigate the neuroprotective effect of Wnt, we measured the Wnt family protein level in QUIN-induced excitotoxicity after Box5 treatment. We found the level of Wnt signaling was increased with Box5 pretreatment, especially, Wnt4, Wnt5a, Wnt7a/b, and Wnt10a, which are known as non-canonical Wnts. Interestingly, there was no change in canonical Wnts, such as Wnt1, Wnt2, and Wnt3 with QUIN or Box5.

There are two different non-canonical Wnt signaling pathways, increasing of Ca2+/PKC pathway and activating the JNK level. To determine the neuroprotective mechanisms of Box5, we investigated the levels of protein, especially the mitogen-activated protein (MAP) kinase family, in QUIN-treated cells using western blotting. The MAP kinase family is known to regulate diverse biological processes, including cell survival and apoptosis, and is comprised of three subfamilies, namely ERK, JNK, and p-38 MAP kinase.

As such, we examined the possible involvement of the PI3K, PKC, ERK, JNK, p38 MAP kinase, and GSK-3β pathways in the effect of Box5 on excitotoxicity. Following the western blot analysis (Figure. 4), QUIN treatment reduced the immunoreactivity of phosphorylated ERK1/2 (p-ERK1/2), whereas Box5 pretreatment elevated the immunoreactivity of p-ERK1/2. These findings indicate that Box5 inhibits the QUIN-induced reductions in p-ERK1/2 levels and further increases these levels. In addition, the level of JNK, part of non-canonical Wnt signaling, was increased with Box5 treatment. Interestingly, the phosphorylation of p38 MAP kinase was increased in QUIN-induced excitotoxicity with Box5 pretreatment. These data indicate that non-canonical Wnt signaling regulates the activation of JNK level and the phosphorylation of p38 MAP kinase in Box5 pretreatment to regulate cell survival.

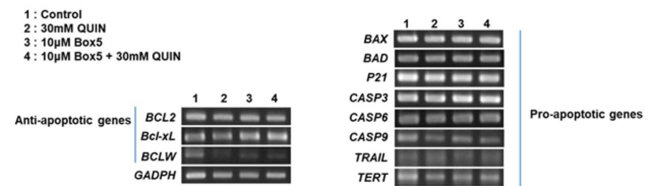

Figure 3. Modulation of anti-apoptotic and pro-apoptotic genes by QUIN and Box5. The cells were incubated with QUIN and Box5 for six hours. mRNA expression for anti-apoptotic genes (BCL2, Bcl-xL, BCLW) and pro-apoptotic genes [BAX, BAD, P21, CASP3, CASP6, CASP9, TNF-related apoptosis-inducing ligand (TRAIL), telomerase reverse transcriptase (TERT)] was analyzed using RT-PCR. The pro-apoptotic gene expression was reduced by Box5 pretreatment, whereas the expression of anti-apoptotic genes was elevated. The RT-PCR assay was repeated five times independently in different cells and representative data are shown. GAPDH was used as a control.

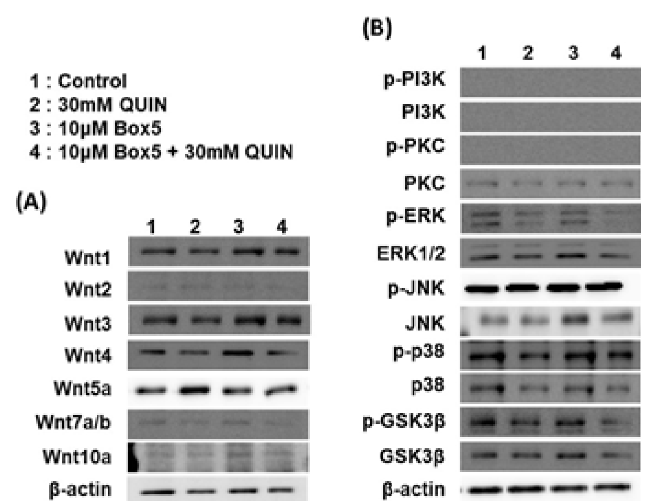

Figure 4. Expression of Wnt signaling-related proteins and Wnt downstream pathway proteins. (A) Western blot analysis demonstrated the effect of Wnt signaling in neuroprotection via Box5 in QUIN-induced excitotoxicity. (B) We demonstrated whether the MAPK pathway, which is known as a Wnt downstream pathway, is regulated by QUIN and Box5 in neuronal cells. The western blot assay was repeated five times independently in different cells and representative data are shown. β-actin was used as a control.

Discussion

In the last few decades, there have been many studies on the role of Wnt signaling in cell survival, Wnt is also thought to regulate apoptosis. Thus, understanding the mechanisms of Wnt signaling and down-stream pathways is important. In addition, Wnt pathways are highly conserved with crosstalk in development and diseases and may play an important role in the neuroprotective response after cell death via diverse protective mechanisms (19).

In this study, we found that the Wnt antagonist, Box5, had a neuroprotective effect on QUIN-induced excitotoxicity through two distinct mechanisms; regulation of the Wnt pathway and activation of cellular signaling mechanisms. An MTT assay and DAPI staining showed Box5 protected the N18D3 cells from apoptotic death. We also demonstrated that Box5 prevented QUIN-induced pro-apoptotic gene expression and increased that of antiapoptotic genes through increased immunoreactivity of ERK and Wnt signaling.

Modulation of the Wnt pathways implicates metabolic pathways in neurodegenerative disease, and Wnt deregulation leads to AD and PD (20). In terms of cell signaling responses, post-translational modifications result in cell death, apoptosis, and neurodegenerative diseases. Phosphorylation, a post-translational modification, occurs during oxidative stress and mitochondrial dysfunction and induces neuronal apoptosis. In addition, nitrosylation from Parkin or cyclin-dependent kinase 5 (Cdk-5) induced the up-regulation of caspase expression and misfolded protein levels, and increased neuronal cell death (7).

Excitotoxicity, a result of extensive neuronal damage, leads to neuronal cell death and acute and chronic neurodegenerative diseases. In this study, QUIN treatment significantly induced neuronal death as shown by the

MTT assay and DAPI staining showing DNA degradation and nuclear condensation. However, the Wnt antagonist, Box5, improved cell viability and reduced DNA fragmentation after 24 hours. Recent evidence indicates that the Wnt signaling pathway may be a new pathological mechanism leading to various neurodegenerative diseases (2, 3, 21). In the clinical field, the Wnt-ON or Wnt-OFF systems in the human brain are regulated by crosstalk with MAPK signaling in response to oxidative stress, apoptotic signals, aging, and cell death (21, 22).

Our data are consistent with other studies demonstrating that Wnt might be a potential therapeutic agent because it regulates the down-stream MAPK pathway.

In recent years, multiple approaches demonstrated the multifunctional MAPK signaling, including the neuroprotective effect of Wnt signaling in drug-induced cell death. However, the mechanisms and pathways responsible for the rescuing effect are still unknown. Here, we demonstrated that the crosstalk between Wnt signaling and the down-stream MAPK pathway might be a key modulator for understanding medical treatment in neurodegenerative diseases.

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Interest conflict

Conflict of interest exists when an author (or the author's institution) has financial or personal relationships that inappropriately influence (bias) his or her actions (such relationships are also known as dual commitments, competing interests, or competing loyalties).

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article

Authors' contribution

Conceptualization, S.J. and J.H.; Methodology, J.H., M.R., H.S.J., and S.J..; Software, J.H. and S.J.; Validation, H.S.J; Formal Analysis, J.H., M.R., H.S.J, and S.J.; Investigation, B.C.K., S.J. and H.S.J; Resources, B.C.K., H.S.J. and S.J.; Data Curation, S.J.; Writing – Original Draft Preparation, S.J.; Writing – Review & Editing, H.S.J. and S.J.; Visualization, J.H. and S.J.; Supervision, B.C.K. and H.S.J; Project Administration, S.J.; Funding Acquisition, J.S.P., H.S.J., and S.J.

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Ethics approval and consent to participate

No human or animals were used in the present research.

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