Original Article

Possible Role of *Porphyromonas gingivalis* in the Regulation of *E2F1*, *CDK11*, and *iNOS* Gene Expression in Neuronal Cell Cycle: A Preliminary Study

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ABSTRACT

Objective: This study aimed at evaluating the *in vitro* effect of *Porphyromonas gingivalis* exposure in gene expression of *E2F1* (family of transcription factors), cyclin-dependent kinase-1 (*CDK11*), and inducible nitric oxide synthase (iNOS) of the neuronal cell cycle. **Materials and Methods:** The culture of neuronal cell line SH-SY5Y was exposed to *P. gingivalis* ATCC 33277, and the gene expression of *E2F1*, *CDK11*, and iNOS was analyzed by using a real-time polymerase chain reaction. **Results:** It was shown that *E2F1*, a G1 phase biomarker and transcription factor, was upregulated in neuronal cells exposed to *P. gingivalis* compared with that in control cells. However, *CDK11*, a biomarker of G2/M checkpoint and iNOS, was downregulated in neuronal cells exposed to *P. gingivalis* compared with that in control cells. **Conclusions:** *P. gingivalis* can regulate the neuronal cell cycle, as indicated in the *E2F1*, *CDK11*, and *iNOS* gene expression.

Keywords: *CDK11, E2F1, iNOS, neuron, Porphyromonas gingivalis*

Introduction

Porphyromonas gingivalis not only plays a role in the development of periodontitis but is also associated with the development of neurodegenerative diseases. Patients with neurodegenerative disorders have been reported to show an upregulation of proteins involved in the neuronal cell cycle. Recently, several studies have reported a high correlation between periodontitis and dementia.[1-3] Individuals who do not brush their teeth everyday have been reported to be at a 22%–65% greater risk of developing dementia than those who brush their teeth thrice a day, and it is known that poor oral hygiene is a risk factor for periodontitis.[4]

In experimental animal models, *P. gingivalis* has been show to produce virulence factors such as gingipain and lipopolysaccharides that destroy host tissues and avoid the host defense system.[5] Gingipain plays a role in the development of periodontitis and is associated with neurodegenerative diseases, such as Alzheimer's disease. A previous study reported that the inhibition of gingipain expression has a therapeutic effect in preventing nerve degradation and accelerates recovery.[6,7] Studies have shown that exposure to lipopolysaccharides produced by *P. gingivalis* contributes to the formation of amyloid-β molecules that are involved in the development of Alzheimer's disease.[8-10] In addition to virulence factors, the DNA of *P. gingivalis* has also been detected in the cerebrospinal fluid of patients with Alzheimer's disease, thus providing further evidence that *P. gingivalis* infection impacts the central nervous system.[11,12]

The idea that the activation of a distorted neural cell cycle might be an essential step in neural death, seen in Alzheimer's disease, arose in the mid-1990s.[13] Research has shown that the brains of patients with

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neurodegenerative disorders show increased expression of proteins involved in the neural cell cycle. An upregulation of neural cell cycle regulators such as *E2F1* was observed in the spinal motor neural and postmortem motor cortex in patients with amyotrophic lateral sclerosis. The reentry of neural cells into the cell cycle is thought to be via an apoptotic pathway that causes neural death.[12,13] *E2F1* is a biomarker of the G1 phase and a transcription factor that can trigger cell cycle activation and apoptosis. *CDK11* is known to be involved in cell cycle control at the G2/M checkpoint.[14] However, the incidence of successful mitosis in patients with Alzheimer's disease has not yet been reported.[15] It has also been reported that *iNOS*, involved in nitric oxide (NO) production, can stimulate stem cell proliferation and induce cell division.[16] However, several other studies have shown that NO can inhibit neural stem cell proliferation under physiological conditions.[17-19] Findings associated with the role of NO are still being debated and require further investigation.

The presence of virulence factors and *P. gingivalis* has been previously reported in patients with Alzheimer's disease,[11,20] and one of the pathways that has been reported to be activated is apoptosis.[21,22] However, the effect of *P. gingivalis* on the neural cell cycle control system has not yet been investigated. This is of interest because if *P. gingivalis* influences the neural cell cycle, it can be harnessed to formulate preventive strategies before extensive neural damage occurs. Thus, in this study, we investigated the effects of *P. gingivalis* exposure on the neuronal cell cycle. Here, we hypothesize that *P. gingivalis* plays a role in regulating the gene expression of *E2F1*, *CDK11*, and *iNOS* in the neuronal cell cycle.

Materials and Methods

Bacterial culture

P. gingivalis ATCC 33277 was cultured on brain heart infusion agar (Merck, Singapore) as the growth medium, and it was incubated under anaerobic conditions at 37°C for 24 h. The cells were transferred to BHI broth, and they were further incubated under anaerobic conditions at 37°C for 24 h.

Neuronal cell culture

SHSY5Y cells (Elabscience, Catalog no.: EP-CL-0208) were cultured in Dulbecco's Modified Eagle's medium with high glucose and L-glutamine (Gibco), 15% fetal bovine serum (Gibco), and 1% Antibiotic-Antimycotic (Gibco). The medium was replaced with fresh media every two to three days. On reaching 80% density, cells were harvested and resuspended in 2 mL of medium.

P. gingivalis exposure of neuronal cells

A 96-well cell culture plate was plated with 4×10^4 neural cells per 100 µL medium per well. Cells treated with 30 µL of 8×10^5 CFU/mL culture of *P. gingivalis*, equivalent to a multiplicity of infection of 20, formed the experimental group, whereas untreated cells were designated to the control group. Each group had six replicates. Cells were incubated for 24 h at 37°C, and they were then harvested for RNA extraction.

RNA extraction and real-time polymerase chain reaction

The RNA was extracted from cell pellets using an RNA extraction kit (GENEzol, 221, Taiwan, New Taipei City). The resultant RNA from the samples was pooled for each group and was used to synthesize cDNA by using a reverse transcription kit (Toyobo, New York) according to the manufacturer's instructions. The obtained cDNA was stored at −80°C until further analysis. The real-time polymerase chain reaction was carried out in duplicates by using the SYBR Premix Ex Taq Kit (Toyobo, New York). The relative expression of the target genes was normalized to that of *GAPDH* and analyzed by using the 2-ΔΔCt method. The primer sequences used are as follows: *E2F1*, forward: 5′-GGGGAGAAGTCACGCTA TGA-3′, reverse: 5′-CTCAGGCACAGGAAAACAT-3′; *CDK11*, forward: 5′-CAGTCTTCAGGA TGTGCTTAT-3′, reverse: 5′-TGACCA GGAGGGATAGAAT-3′; *iNOS*, forward: 5′-CAGAATGTGAC CATCA TGG-3′, reverse: 5′-ACAACCTTGGTGTTGAAGGC-3′; *GAPDH*, forward: 5′-CTGCAC CACCAACTGCTTAG-3′, reverse: 5′-AGGTCCACCACTGACACGTT-3′.

Results

The RT-qPCR melting temperature (T_m) curve of *E2F1* gene amplification showed two peaks at ranges of 75°C–76°C and 81°C–82°C [Figure 1A], and this is an indication of stable amplicon regions, as they do not melt immediately and remain in the double stranded form until the temperature becomes high enough to melt it.[26] It was also observed that neuronal cells exposed to *P. gingivalis* showed a higher expression of *E2F1* than that in the control cells [Figure 1B].

The melt curve of *CDK11* gene amplification showed a single peak with a T_m of 77.54°C [Figure 2A], which indicates the specificity of target gene amplification.[27] In contrast to *E2F1* expression, *CDK11* expression in neuronal cells exposed to *P. gingivalis* bacteria was lower than that in control cells [Figure 2B].

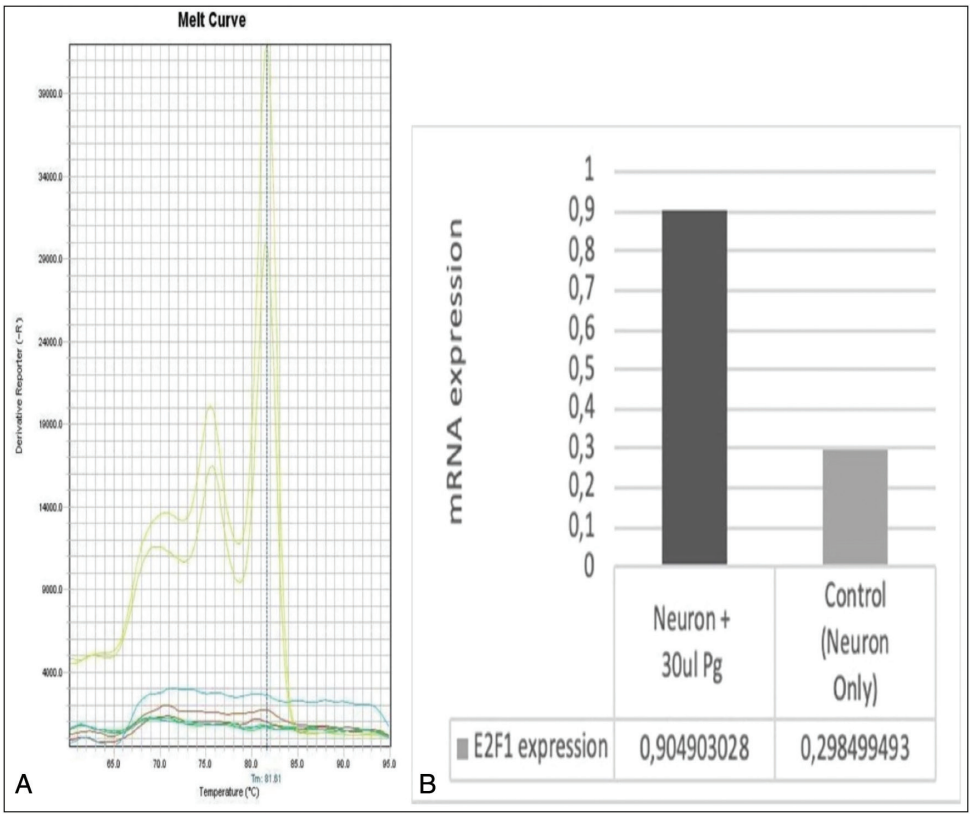

Figure 1: Results of *E2F1* real-time polymerase chain reaction gene analysis. (A) Melt curve for *E2F1*; (B) *E2F1* mRNA expression

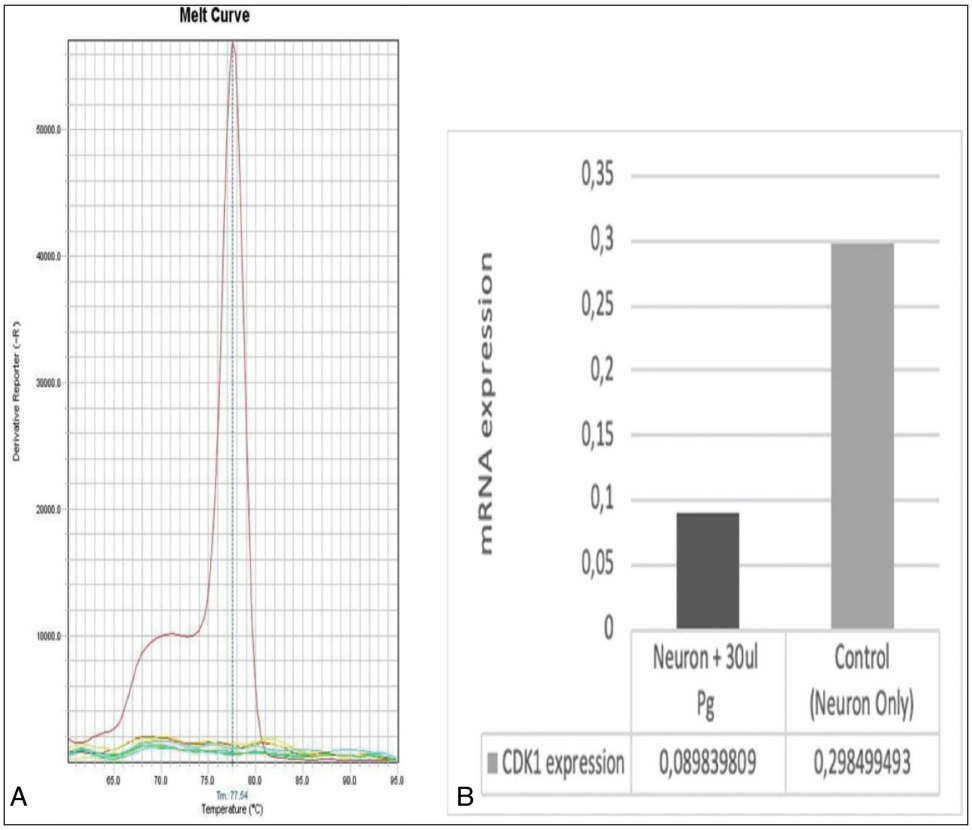

Figure 2: Results of *CDK11* real-time polymerase chain reaction gene analysis. (A) Melt curve for *CDK11*; (B) *CDK11* mRNA expression

The melt curve of *iNOS* gene amplification showed the existence of multiple peaks with T_m values of 78°C–80°C, 84°C, and 88°C [Figure 3A]. Additional sequential factors may cause this, such as a secondary structure in the amplicon that causes it to melt in several phases.[26] The expression of *iNOS* in neuronal cells exposed to *P. gingivalis* was lower than the *iNOS* expression in control cells [Figure 3B].

Discussion

In this study, we analyzed the effects of *P. gingivalis* exposure on the neuronal cell cycle by estimating the expression levels of molecules that play a role in the cell cycle control system, such as *E2F1*, *CDK11*, and *iNOS*.

It is known that *E2F1* acts as a transcription factor to initiate gene transcription that is essential for the cell cycle. *E2F1* also induces cell death under pathological conditions by activating the transcription of apoptosis-related genes. Under conditions of potassium deficiency, *E2F1* causes the apoptosis of cerebellar granule cells, by modulating the expression of CDC2, as well as other apoptosis-related genes. *E2F1* also acts as a transcriptional activator in the G1/S phase of the cell cycle in human cells.[14,21] In this study, neuronal cells exposed to *P. gingivalis* show a higher expression of *E2F1* than control cells. Thus, we speculate an *E2F1*-mediated reactivation of the neuronal cell cycle. In the study by Folch *et al.* (2012), the reactivation of the cell cycle was shown to trigger apoptosis.[13]

Based on a previous postmortem study, post-mitotic neural cells exist in the G2/M phase. Biomarkers of the G2 phase include *CDK11*, whereas those of the M phase are cyclin B and *CDK11*. However, differentiated neuronal cells in patients with Alzheimer's disease are speculated to not complete the cell cycle and a successful completion of mitosis in patients with Alzheimer's disease has not been reported. Therefore, it has been hypothesized that although post-mitotic neural cells exist in the G2/M phase, they are prevented from developing further in patients with Alzheimer's disease.[15] Previous reports have found that *CDK11* is expressed in post-mitotic neuronal cells, with naturally occurring periods of cell death, in mammalian brains. Distorted expression of *CDK11* and cyclin B1 has also been reported post-mortem, in parts of the human brain from various neurodegenerative diseases, suggesting that *CDK11* may be involved in the pathological and developmental neuronal cell death.[28] This study indicates that the expression of *CDK11* in neuronal cells exposed to *P. gingivalis* is lower than that in control cells. Therefore, we hypothesize that neuronal cells that have been infected by *P. gingivalis* cannot complete the cell cycle to the G2/M checkpoint. This finding is consistent with van Leeuwen and Hoozemans's report

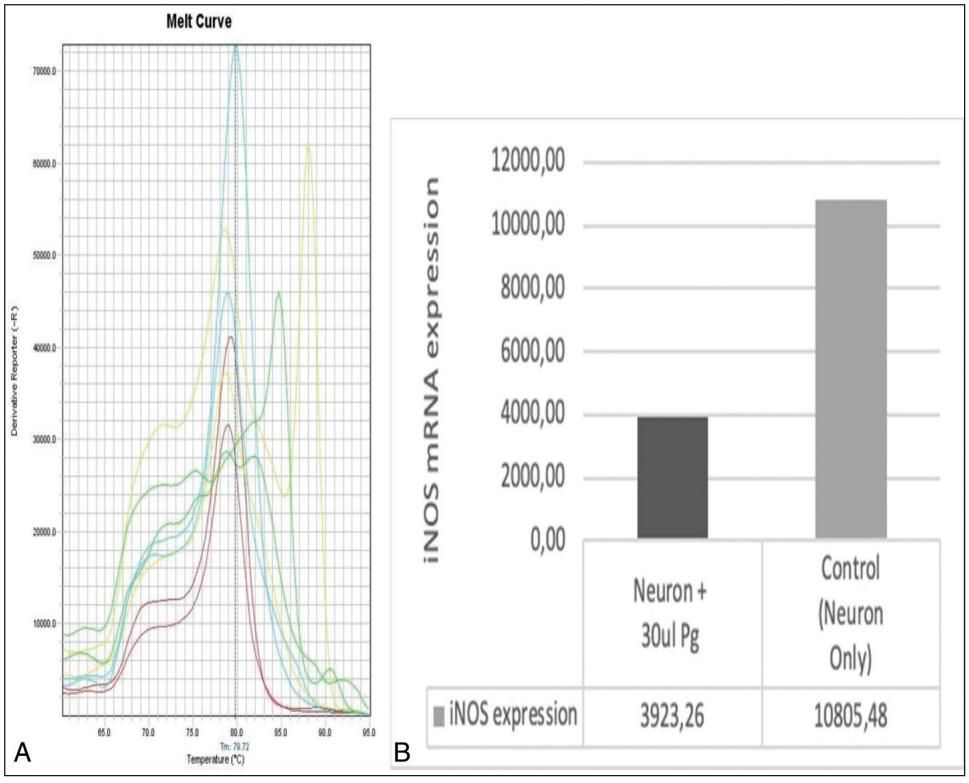

Figure 3: Results of *iNOS* real-time polymerase chain reaction gene analysis. (A) Melt curve for *iNOS*; (B) *iNOS* mRNA expression

(2015), which states the absence of successful mitosis in patients with Alzheimer's disease.[15]

The role of NO in the cell cycle remains unclear. In physiological conditions, it is stated that NO derived from the activity of iNOS can inhibit neuronal cell proliferation.[17-19] In contrast, research suggests that after a brain injury, NO leads to an increase in proliferation and the neurogenesis of neuronal stem cells. Other studies also support the fact that NO is produced during brain injury-associated inflammation to promote cell proliferation. However, the signaling pathways involved in the proliferative effects of NO after brain injury remain unknown.[16,29] In this study, *iNOS* expression in neuronal cells exposed to *P. gingivalis* was lower than that in control cells. This is in contrast with Carreira *et al.*'s (2010) and Zhu *et al.*'s (2003) findings, which state that infected neuronal cells show an increase in NO and cell proliferation.[16,29] This difference might be due to differences in neuronal cell lines. In Carreira *et al.*'s study, the cells cultured were from the subventricular zone in mice, whereas in Zhu *et al.*'s study, the cells were from the rat dentate gyrus.[16] However, according to Packer *et al.* (2003), Moreno-López *et al.* (2004), and Matarredona *et al.* (2005), NO can inhibit cell proliferation under physiological conditions.[17-19] Thus, low expression of NO can increase cell proliferation. This phenomenon is also consistent with the increase in *E2F1* expression in neuronal cells exposed to *P. gingivalis.*

We used *P. gingivalis* ATCC 33277 in this study. Naito *et al.* (2008) stated that this strain was less virulent than *P. gingivalis* strain W83.[30] Therefore, in this study, it is possible that the capacity of *P. gingivalis* bacteria to infect neuronal cells is low. In future studies, we hope to investigate the effect of more virulent strains on neuronal cells. Further research is also needed to determine the underlying mechanism and virulence factors of *P. gingivalis* that influence the neural cell cycle control system.

Thus, *P. gingivalis* infection can reactivate the neuronal cell cycle via the increase in the expression of G1 phase biomarker and transcription factor, *E2F1*. However, most neuronal cells exposed to *P. gingivalis* cannot complete the cell cycle after the G2/M checkpoint because of the decrease in the expression of *CDK11*.[23,24] *P. gingivalis* can also increase neural cell proliferation via a decline in *iNOS* expression, as the NO produced by iNOS is known to inhibit cell proliferation.[25] Although this study has some limitations, such as very limited samples were used, our findings motivate future research to find a strategy to prevent neuronal damage caused by *P. gingivalis* infection. The potential strategy to prevent neuronal damage is to use antibodies, especially anti-*P. gingivalis* antibody, to suppress factors that can damage neuronal cells. Developing a *P. gingivalis* vaccine or other antimicrobial peptides might be beneficial since it is one of the oral bacteria that trigger Alzheimer's disease.

In conclusion, *P. gingivalis* can upregulate *E2F1* and downregulate *CDK11 and iNOS* gene expression in the neuronal cell cycle.

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Conflicts of interest

There are no conflicts of interest.

Authors' contributions

EWB: Research design, supervision, proofread of the manuscript; TRS: Laboratory work, data analysis, manuscript drafting.

Ethical policy and institutional review board statement

Not applicable.

Patient declaration of consent

Not applicable.

Data availability statement

Data available by email to EWB.

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