BRIEF REPORT

REVISED Expression of *TNF*, *IL1B*, and *iNOS2* in the neural cell after induced by *Porphyromonas gingivalis* with and without coating antibody anti-*Porphyromonas gingivalis* [version 4; peer review: 2 approved]

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Abstract
Porphyromonas gingivalis has virulence factors such as gingipain and lipopolysaccharide, causing bacteremia to reach the brain and activate neuroinflammatory release cytokines. This study analyzed the effect of the co-culture of neuron cells with *P. gingivalis* coated with anti-*P. gingivalis* antibodies against cytokines produced by neuron cells. The gene expressions of the *TNF*, *IL1B*, *iNOS2* in neurons was evaluated using RT-qPCR. The results showed that *P. gingivalis* coated with anti-*P. gingivalis* antibody before co-culture with neuron cells could decrease the gene expression of *TNF*, *IL1B*, and *iNOS2* of neuron cells.

Keywords
Porphyromonas gingivalis, Blocking Antibody, Neuroinflammation, TNF-α, IL-1β, iNOS

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REVISED **Amendments from Version 3**

I would like to inform you that we have revised the manuscript according to the reviewer's suggestion in 211–213 page as follows: 'The limitation of using 96 well plate cultures is that the cell number in each well is a small amount. Therefore, a future study using 24 well plate cultures is needed to get more appropriate RNA samples to be analysed'.

Any further responses from the reviewers can be found at the end of the article

Introduction

Periodontitis is an infectious disease that causes inflammation of the tooth-supporting tissue, loss of bone adhesions, initiated by the main pathogens, *Porphyromonas gingivalis*. These bacteria are Gram-negative and have virulence factors such as fimbriae, gingipain, and lipopolysaccharide (LPS), which play a critical role in inducing periodontitis. With this virulence factor, *P. gingivalis* and its products not only damage the periodontal tissue but can also enter the blood circulation or bacteremia and cause systemic spread[1,2]. *P. gingivalis* can move to other organs such as the heart and brain. Sophie's research found the presence of LPS *P. gingivalis* in the brains of Alzheimer's patients[3]. The mechanism for invading *P. gingivalis* bacteria into brain tissue is by penetrating the blood-brain barrier and damaging neuron cells[4]. When entering the central nervous system, these bacteria will first activate defense cells in the brain, namely the microglia, and astrocytes. Activation of both then releases neuroinflammatory mediators such as TNF-α and IL-1β. Several studies have stated that neuron cells themselves can also release the neuroinflammatory mediators TNF-α and IL-1β triggered by foreign bodies such as bacteria. This excessive release of neuroinflammation is toxic to neuron cells and can cause their damage and death[5,6]. Besides, the excessive release of inducible nitric oxide synthase (iNOS) molecule due to antigen by neuron, microglia, and astrocyte cells, may induce human brain neurodegeneration[7].

As a form of defense against bacterial attack, the body will naturally produce antibodies to eliminate bacteria. The antibodies produced by the host can specifically recognize certain bacterial species. Either monoclonal or polyclonal antibodies can recognize the lipid A region of the LPS of Gram-negative bacteria, such as *P. gingivalis*[8]. Animal studies by Barekzi *et al*. stated that pooled human polyclonal antibodies that are injected locally in the area of injury in mice have broad-spectrum antimicrobial effects against Gram-negative bacteria[9]. *P. gingivalis* reside in a structured community of biofilm attached to surfaces embedded in the extracellular matrix which they produce themselves and they are difficult to eradicate due to their resistance to antimicrobials and the body's defense mechanisms[10]. The passive immunization approach using polyclonal antibodies to inhibit *P. gingivalis adhesion* to the periodontium tissue is a strategy to prevent biofilm formation and periodontium tissue damage which can lead to deeper tissue invasion so that *P. gingivalis* can enter the systemic circulation. This study aims to evaluate the effect of anti-*P. gingivalis* antibodies on *TNF*, *IL1B*, and *iNOS* gene expression when bacteria interact with neuron cells. We hypothesized that there are differences in the gene expression of *TNF*, *IL1B* and *iNOS* in SHSY-5Y cells that have been exposed to *P. gingivalis* with and without antibody coating.

Methods

Cell lines

This research is an experimental laboratory study with post test only control group design. This study used the neuron cell line SHSY-5Y (Elabscience, USA), originating from a four-year-old human's bone marrow neuroblastoma. The cell culture medium was DMEM High Glucose with L-glutamine (Caisson Labs, USA), 15% FBS (Gibco, South America), and 1% Antibiotic-Antimycotic (Gibco, USA). The cultured condition was 5% CO2 at 37°C incubator until 90% confluency was achieved (Figure 1)[11].

Porphyromonas gingivalis ATCC 33277 was cultured in Brain Heart Infusion (BHI) agar as a growth medium and incubated under anaerobic conditions with a temperature of 37°C for 24 hours. Then cultured into BHI broth and incubated again under anaerobic conditions with a temperature of 37°C for 24 hours. Then stored at 4°C until ready to use.

This study also used *P. gingivalis* ATCC 33277 bacterial culture. The multiplicity of infection (MOI) used was 1:100, the number of bacteria was 3.6×10^7 CFU/mL, and the number of neuron cells was 8×10^5 cells/well. In addition, this research used serum anti-*P. gingivalis* antibodies obtained from rabbits after immunization of killed *P. gingivalis*. *P. gingivalis* antisera were obtained from one-month-old rabbits that have been immunized with 1 mL of 1.7×10^8 CFU/mL *of P. gingivalis* culture. The bacteria were inactivated at 60°C for 30 min before being injected intravenously to the rabbit for 8 weeks with two boosters in intervals of 2 weeks. The animals were euthanized by anesthetic ether inhalation and injection by overdose of anesthetic drug (ketamine 50 mg/kg IM and xylazine 10 mg/kg IM), which caused the animal to fall asleep then slowed and eventually stopped the heart. The blood serum was determined by agar gel precipitation test (AGPT) and the antibody was purified using the Qiagen (QIAGEN, Inc., Valencia, Calif.) protein purification kit, following the manufacturer's protocol. Ethical clearance was given by the Ethical Research Committee of Medical Faculty Universitas Indonesia (2020, number 19-11-1402).

Coating of anti-*P. gingivalis* antibodies

The antisera coated *P. gingivalis* (3.6×10^7 CFU/mL) was prepared by 1:300 diluted rabbit antibody serum in 150 µL growth medium (DMEM High Glucose with L-glutamine (Caisson Labs, USA), 15% FBS (Gibco, South America), and 1% Antibiotic-Antimycotic (Gibco, USA)) for the treatment group; the control was *P. gingivalis* (3.6×10^7 CFU/mL) in 150 µL growth medium and the growth medium only without addition of bacteria. The tubes were then incubate for 1 hour in an incubator with a temperature of 37°C[12].

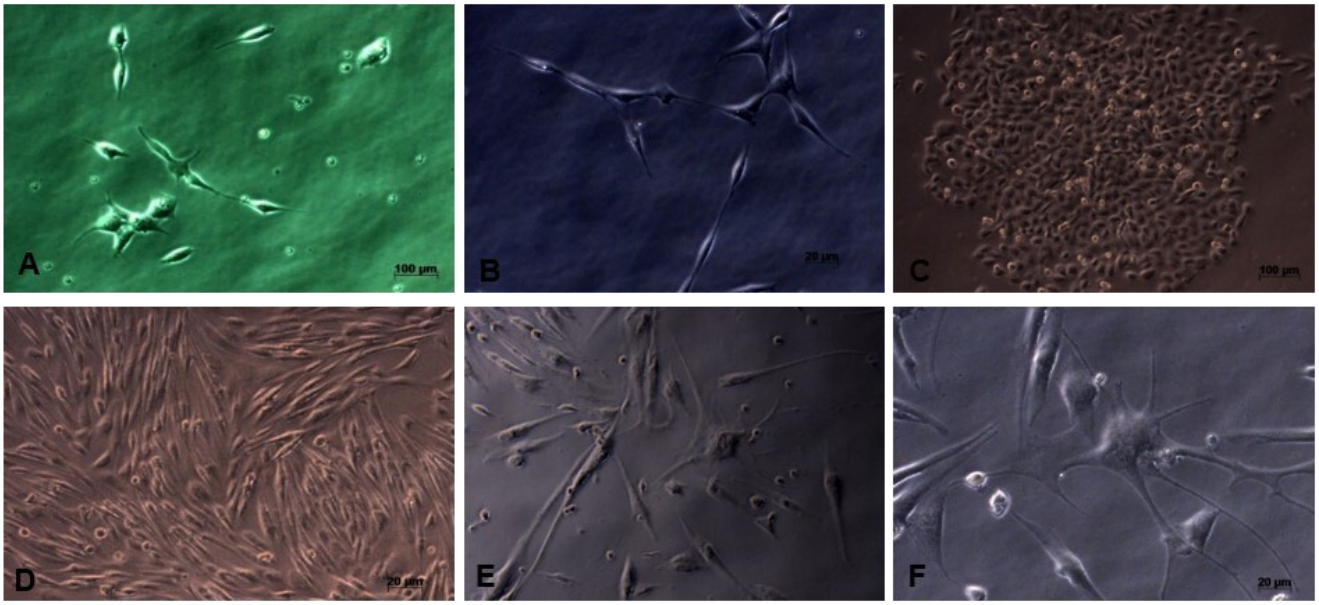

Figure 1. The appearance of the cultured neuron cell line SHSY-5Y; it appears that the SHSY-5Y cells have a neuronal-like cell shape. (**A**) Cell image of 3 days culture (40x enlargement). (**B**) Cell image after 7 days, showing elongation of neuron cell bodies and cells growing in groups (40x magnification). (**C**) There is an increase in cell proliferation and group cell growth (20x magnification). (**D**) The cells have reached 80% confluence and are ready to be harvested (20x magnification), (**E** and **F**) SHSY-5Y cells have undergone differentiation, seen the presence of axons from cells and the cell proliferation process begins to decline (40x magnification). *Underlying data* shows raw, unprocessed images used to generate this figure[13].

Experimental design

The experiment design as follows: group A was the neurons plus bacteria with antibody coating, and group B for the neuron group plus bacteria without antibody coating and medium only, with 6 replications of each group.

Harvest of SHSY-5Y neuron cells

Neuron cell cultures that had reached 80% confluence were harvested using 0.25% trypsin-EDTA (Gibco, Canada). The number of cells harvested was counted using a hemocytometer (number of cells 8×10^5 cells/well). The cells were then transferred to a 15 mL tube and resuspended in 2 mL growth medium and then divided into well plates that have been designed with each well containing 100 µL (4×10^3 cells/well) of SHSY5Y cells. The neuron cell line SHSY-5Y (Elabscience, USA) is a cell that has epithelial-like cell and neuronal-like has a cell density of more than 1×10^6 cells/cm^2. In this study, cell culture was carried out with two subcultures in January 2020 and February 2020 until the number of cells reached 8×10^5 cells/well. Observation with a microscope was carried out every 2–3 days to identify neuron cells and determine the stage of neuron cell differentiation (Figure 1)

P. gingivalis exposure to SHSY-5Y cells

Each well of 96 well culture plate filled with SHSY-5Y cells and antibody-coated *P. gingivalis* bacteria and incubated for one hour were added. Group A was filled with 30 µL (1×10^5 CFU/mL) *P. gingivalis* coated with antibody, while group B was filled with 30 µL of bacterial *P. gingivalis* without antibodies. After that, cells were incubated for 24 hours at 37°C. All cells in the well plate were then harvested for RNA extraction.

RNA extraction and RT-qPCR

The neural cell culture was harvested, and RNA extracted for cDNA synthesis using a Reverse Transcription Kit (ReverTra Ace®, Toyobo, Japan) in line with the manufacturer's instructions. The pooled cDNA sample is ready for use in the Real-Time PCR tool, with the selected primers as Table 1. RT-PCR was performed using the SYBR Premix Ex Taq TM kit. Relative expression of the target gene normalized to GAPDH, gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method and compared to control. The gene expression of *TNF*, *IL1B* and *iNOS* were evaluated by RT-qPCR as previously reported[14]

Results

Figure 2 shows the SHSY-5Y cells that were not exposed to *P. gingivalis*, and those exposed to *P. gingivalis* and coated with anti-*P. gingivalis* antibodies. From these figures, it is known that cells not exposed to *P. gingivalis* grew more than cells exposed to *P. gingivalis*, both with and without antibodies.

From qPCR analysis, it was observed that there are differences in the gene expression of TNF-α, IL-1β and iNOS in

Table 1. Primers used in this study.

Primer Name	Sequences	Reference
TNF	Forward: 5 'CTG AAC TTC GGG GTG ATC G 3' Reverse: 5 'GCT TGG TGG TTT GCT ACG AC 3'	15
IL1B	Forward: 5'-TAT TAC AGT GGC AAT GAG G-3 Reverse: 5'-ATG AAG GGA AAG AAG GTG-3'	15
iNOS	Forward: , 5'-GCA GAA TGT GAC CAT CAT GG-3' Reverse: 5'-ACA ACC TTG GTG TTG AAG GC-3	16
GAPDH	Forward: 5'-CTG CAC CAC CAA CTG CTT AG-3' Reverse: 5'-AGG TCC ACC ACT GAC ACG TT-3'	15

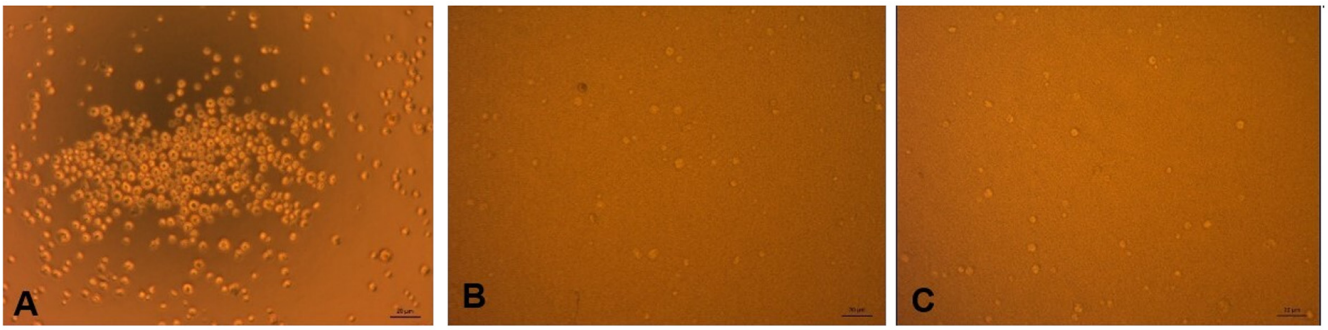

Figure 2. (**A**) SHSY-5Y cell before exposure to *P. gingivalis*, (**B**) After exposure to *P. gingivalis* without antibodies, (**C**) After exposure to *P. gingivalis* with antibodies. (Light microscope, 20x magnification).

SHSY-5Y cells that have been exposed to *P. gingivalis* with and without antibody coating, it can be concluded that the research hypothesis is accepted. This is shown in Figure 3, where the expression of TNF-α and IL-1β genes in the antibody-coated group was lower than in the antibody-coated group. Ct values are available as *Underlying data*[17].

Discussion

The SHSY-5Y neuron cell line (Elabscience, USA) is a cell derived from human neuroblastoma and taken from bone marrow tissue. These cells have epithelial-like cell and neuronal-like cell morphology. During culture, SHSY-5Y cells can grow into two types of cells, namely adherent cells and floating cells, both of which are viable. However, in this study, adherent cells were used because they were clearer in morphology and proliferation development, and were easy to evaluate after a routine medium change[18,19].

Microscopy images of SHSY-5Y cells (Figure 1) showed significant growth changes over time. According to Kovalevich and Langford, one of the considerations for the success of SHSY-5Y cell culture is the growth medium used[19]. In this study, DMEM growth medium containing L-glutamine was used. Glutamine can help increase neuron cell viability and increase neuron cell density, so that it can be seen on microscopy images that neuron cell cultures grow well. However, the number of cells collected until the end of cell culture is 8×10^5, where this number is limited for the study sample. This may occur because cells have started to enter the differentiation stage, so that the cell proliferation process tends to decrease[18]. Based on direct observation under a microscope, the results of Figure 2 data show that the growth in the number of cells does not differ between coating antibody and without coating antibody. It is likely that if we observed using a viability test such as the MTT test (MTT 3- (4,5-dimethyltiazol-2-yl) -2,5-diphenyltetrazolium bromide), the cell count would be able to be counted. Another possibility is that the number of cells did not differ, but the cell's metabolism changed between experimental groups, characterized by differences in the mRNA expression of neuroinflammatory cytokine.

TNF-α and IL-1β are inflammatory mediators released by immune cells when a stimulus triggers the cells. In the nervous system, TNF-α and IL-1β are usually released by astrocytes and microglia cells. However, a number of studies suggest that these inflammatory mediators are also released in large numbers by neuron cells when there are intrinsic or extrinsic triggers[20]. Extrinsic triggers such as LPS presence from *P. gingivalis* bacteria can trigger the expression of TNF-α and IL-1β by neuron cells so that it can damage neuron cells[21–23]. In the incidence of Alzheimer's disease, the release of this inflammatory mediator can cause neuronal cell death, according

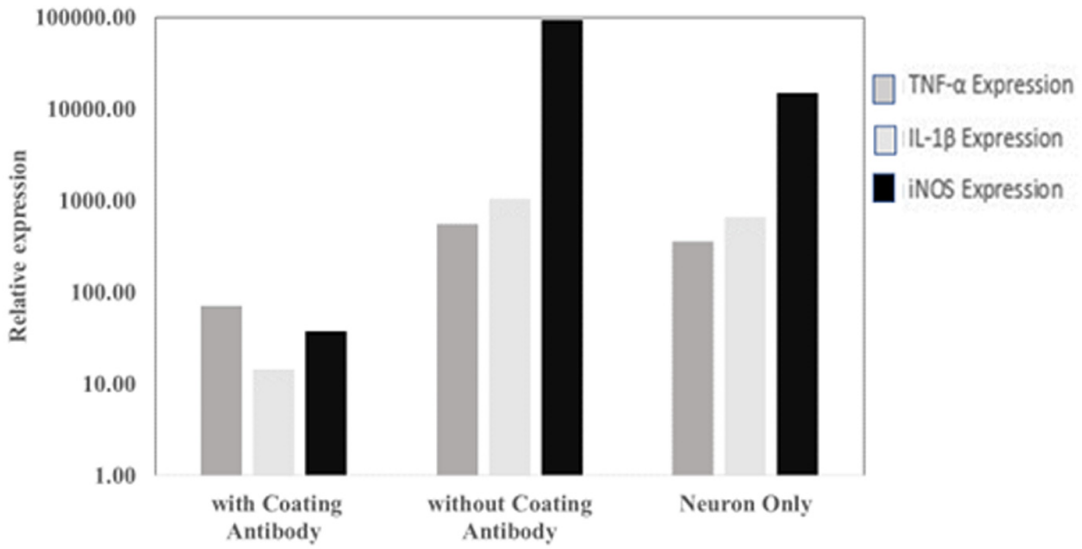

Figure 3. The level of *TNF*, *IL1B* and *iNOS* gene expression in the antibody-coated group, without antibody coating and neuron cells only.

to a study by Janelsins *et al*., which stated that the inflammatory mediators TNF-α and IL-1β appears to be directly proportional to Alzheimer's disease severity[23–25].

This study is in line with the research of Janelsins *et al*., who found that neuron cells can express TNF-α in brain injury in experimental animals. This is evidenced by the detection of the molecules NeuN and TNF-α in the brain of six-month-old mice. In this study, SHSY-5Y neuron cells can also express TNF-α. In addition, Janelsins *et al*. also found that TNF-α contributed to neuron cell death in the brain with Alzheimer's condition. The signaling mechanism is still unknown, but Janelsins *et al*. stated that there was an increase in the expression of TNFRII and Jun transcript as pro-apoptotic signals mediated by TNF-α[25,26].

The expression of iNOS has been characterized in various cell types as an inflammatory mediator during infection, disease, or tissue damage. INOS is expressed by astrocytes, microglia, and a small portion of endothelial cells in the brain. However, under conditions of increased inflammatory activation in neuron cells, neurons can also express these cytotoxic agents and other reactive oxidative species. The main component that regulates the signaling pathway of iNOS in neurons is the transcription factor NF-κb. The results of this study indicate that anti-*P. gingivalis* antibodies can suppress iNOS expression in neuron cell cultures exposed to *P. gingivalis*. Blocking carried out by antibodies to *P. gingivalis* LPS was thought to suppress bacterial pathogenicity so that iNOS expression in neurons was lower than that of the control group. We assume the antibody use reduced neuronal damage. This is in line with Heneka and Feinstein's research, which states that increased expression of iNOS in neurons can affect neurodegeneration and inflammation in the brain[7,27].

P. gingivalis have secreted and non-secreted virulence factors. Secreted virulence factors, for example, gingipain, are virulence factors secreted by bacteria to carry out their activities. Meanwhile, non-secreted virulence factors are virulence factors that are not secreted by bacteria, usually attached to the bacterial structure, such as LPS. In this study, the antibodies used were from injections of killed *P. gingivalis* in rabbits. This will result in the formation of polyclonal antibodies against non-secreted virulence factors, namely LPS, because when it is turned off, the bacteria are unable to secrete other virulence factors such as gingipain. The anti-*P. gingivalis* polyclonal antibodies can recognize *P. gingivalis* bacterial cells and these bacteria's LPS structure[8,9,26]. Therefore, coating this antibody with *P. gingivalis* bacteria for 1 hour before exposure to neuronal cells is thought to block LPS *P. gingivalis* bacteria not to infect neuron cells.

In contrast to the control group that did not use antibodies, *P. gingivalis* was exposed to neuron cells, infecting neuron cells with secreted and non-secreted virulence factors. This occurs because there are no antibodies that block the two types of *P. gingivalis* virulence factors. Therefore, in qPCR analysis results, neuron cell culture with anti *P. gingivalis* antibody showed lower TNF-α and IL-1β expression than the control group. The study (Figure 3) show that the use of antibodies can suppress the expression of TNF-α and IL-1β. The low expression of TNF-α and IL-1β with the use of antibodies is thought to

prevent neuronal damage and is expected to prevent the occurrence of Alzheimer's disease or other cognitive disorders. However, different research results may occur because of the MOI value used. In this study, the MOI used was 1:100.

The limitation of this study is that the pooled samples method has some biases since the equal amount of RNA was used for each individual sample are not the same and it may cause some alteration of individual RNA contributions such that some samples dominate more than others in the pooled expression[28].

The limitation of using 96 well plate cultures is that the cell number in each well is a small amount. Therefore, a future study using 24 well plate cultures is needed to get more appropriate RNA samples to be analysed.

Although there were some limitations of this study, our findings indicate that there is good potential for the development of the anti-*P. gingivalis* vaccine. The anti-*P. gingivalis* antibody used in this study was able to block the development of bacteria *in vitro* so that the neuroinflammatory response can also be minimized. Further research at the *in vivo* level and clinical trials can be developed to see the positive effects of administering antibodies locally or systemically. In the case of local infection of *P. gingivalis* in the oral cavity, the local administration of antibodies may have more potential to suppress bacterial development.

In addition, long-term research involving the role of neuron cells and damage to the central nervous system also needs to be done. With this research, it is hoped that it can become a reference to increase the level of research so that in the future, the prevention of *P. gingivalis* infection can be done so that it can prevent neurodegeneration in the incidence of Alzheimer's disease.

Conclusion

The cultured SHSY-5Y neuron cells exposed to *P. gingivalis* bacteria after anti-*P. gingivalis* antibody coating exhibited a reduction in the expression of the *TNF*, *IL1B*, and *iNOS*. Further research to see the effectiveness of anti-*P. gingivalis* antibodies still needs to be developed, especially *in vivo*. The success of anti-*P. gingivalis* antibodies in suppressing factors that can damage neuronal cells can be used as a guideline for developing a *P. gingivalis* vaccine, since it is one of the oral bacteria that triggers Alzheimer's disease.

Data availability

Underlying data

Open Science Framework: Expression of TNF-α, IL-1β, and iNOS in the neural cell after induced by Porphyromonas gingivalis with and without coating antibody anti-Porphyromonas gingivalis. https://doi.org/10.17605/OSF.IO/Q5CVW[17].

This project contains the following underlying data:

- Beta actin GAPDH 2506202_data (1).xls. (qPCR data for housekeeping gene *GAPDH*.)
- IL1b TNFa_data(1).xls. (qPCR data for *IL1B* and *TNF*.)
- iNOS 23062020_data.xls. (qPCR data for *iNOS*.)

Open Science Framework: Expression of TNF, IL1B, and *iNOS* in the neural cell after induced by Porphyromonas gingivalis. https://doi.org/10.17605/OSF.IO/JFG3T[13].

This project contains the raw images used to produce Figure 1.

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28. Bachtiar EW, Putri CF, Soejoedono RD, *et al.*: **Peer Review Report For: Expression of *TNF*, *IL1B*, and *NOS2* in the neural cell after induced by *Porphyromonas gingivalis* with and without coating antibody anti-*Porphyromonas gingivalis* [version 2; peer review: 1 approved, 1 approved with reservations].** *F1000Res.* 2021; **9**: 1499.
Publisher Full Text

Open Peer Review

Current Peer Review Status: ✔ ✔

Version 4

Reviewer Report 20 July 2021

https://doi.org/10.5256/f1000research.58087.r88415

✔ **Hadhimulya Asmara**
Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada

I accept the recent revision.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neuroscience (any works with neurons involvement), Molecular Biology (any works related to DNA and protein).

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 29 June 2021

https://doi.org/10.5256/f1000research.58087.r88414

✔ **Widya Lestari**
Department of Oral Biology, Kulliyyah of Dentistry, International Islamic University Malaysia (IIUM), Kuantan, Malaysia

Good and may index as it is.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Biologist

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 3

Reviewer Report 06 May 2021

https://doi.org/10.5256/f1000research.56228.r84063

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Hadhimulya Asmara
Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada

I have questions for the author regarding the amendments from version 2 above. In the last paragraph at the last sentences, the author mentioned "Using 24 well plate cultures will get more appropriate RNA samples to be analyzed in order to overcome this problem and the statistical analysis will be employed."

My questions are:

1. Will the author change the experiments using 24 well plate in order to overcome the problem of analyzing RNA samples?

2. Will the author employ the statistical analysis as mentioned in the sentences?

I hope the author can clarify my questions above.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neuroscience (any works with neurons involvement), Molecular Biology (any works related to DNA and protein).

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 10 Jun 2021

Boy Muchlis Bachtiar, Faculty of Dentistry Universitas Indonesia, Jakarta, Indonesia

Dear reviewer,

I would like to clarify the questions related mention: " The limitation to the use of 96 well

plate cultures is that the number of cells in each well is very limited. Maybe for future research, using 24 well plate cultures will get a more appropriate RNA sample to be analyzed in order to overcome this problem to be able to use statistical analysis".

The reviewer's questions are:

1. Will the author change the experiments using 24 well plate in order to overcome the problem of analyzing RNA samples?

2. Will the author employ the statistical analysis as mentioned in the sentences?

The author's response:
We apologize, maybe the sentence above is confusing. What we mean is for future research the use of well 24 plates. We mean this is a discussion that might be suggested to be applied to future research so that statistical analysis can be applied.

We hope you understand what we mean and we will be happy to wait for your suggestions if there is any need to improve the manuscript.

Thanks in advance,
With warm regards,
BACHTIAR EW

Competing Interests: No competing interests were disclosed.

Author Response 24 Jun 2021

Boy Muchlis Bachtiar, Faculty of Dentistry Universitas Indonesia, Jakarta, Indonesia

Dear Reviewers,

Thanks for your valuable suggestions. I would like to inform you that we have revised the manuscript according to your suggestion in 211-213 page as follows: 'The limitation of using 96 well plate cultures is that the cell number in each well is a small amount. Therefore, a future study using 24 well plate cultures is needed to get more appropriate RNA samples to be analyzed'. Thank you in advance.

Looking forward to hearing from you.
With warm regards
BACHTIAR EW

Competing Interests: No competing interest

Reviewer Report 29 April 2021

https://doi.org/10.5256/f1000research.56228.r84062

Widya Lestari
Department of Oral Biology, Kulliyyah of Dentistry, International Islamic University Malaysia (IIUM), Kuantan, Malaysia

Approved

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Biologist

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 01 April 2021

https://doi.org/10.5256/f1000research.55228.r81562

Hadhimulya Asmara
Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada

Author's responses:
We have deleted 'morphology' In Figure 2, it can be seen that there is a decrease in the number of neuron cells after being exposed to P. gingivalis bacteria.

Comment:
Even though the author has already deleted the term "morphology" in figure 2 and in the figure legend, but in the explanation in the result section (first paragraph), the author still states the term "morphology" which is not represented on figure 2.

Author's responses:
Qualitatively, from the microscope image (Figure 2), there was no difference between the antibody group and the non-antibody group. In our opinion, the number of cells seen on a microscope does not necessarily indicate the expression of neuroinflammation. Based on the results of the real time PCR analysis, it showed that the expression of neuroinflammation was more in the group without antibodies (Figure 3).

Comment:
The author's response does not address the concern of the result in figure 2 that it is against or at least does not support the author's hypothesis. The number of cell growth in the cells that are treated with antibody coated are supposed to be higher than the one without the antibody coated. Yet there is no explanation from the author about this data in the result and discussion sections in correlation with the hypothesis. In my opinion, the author should explain the result of this figure 2 data, why does not this result support the hypothesis and why does the cell numbers growth does not differ between coating and without coating.

Author's responses:
We used pooled samples (we have added this information in the methods section) as there was insufficient amount of RNA from each individual replication of the experiments. But we think the value of gene expression presented here is equal to de average of 6 replications of experiment. Hence, we couldn't get a statistical significance. Some studies also use this kind of data interpretation (Shu-Dong Zhang, Timothy W. Gant, Effect of pooling samples on the efficiency of comparative studies using microarrays, Bioinformatics, Volume 21, Issue 24, 15 December 2005, Pages 4378–4383)

Comment:
The author cannot show the statistical significance or P values of the different expressions of these genes instead using pooled samples to justify the different values of the gene expression. There is a report about the weaknesses of using pooled samples method. According to that report, the pooled samples method has some biases since the equal amount of RNA was used for each individual sample are not the same and it may cause some alteration of individual RNA contributions such that some samples dominate more than others in the pooled expression. Another disadvantage is that one may not be able to associate the gene expression from the pooled sample with the individual phenotypic information, and thus cannot make certain statistical inference or predictions for individuals. Based on those disadvantages, the authors of that report suggested everyone has to be cautious about designing a pooled experiment. They also suggested if there is not enough RNA from each individual sample to run an array, the number of different pools should not be too small and the number of subjects should be appropriately increased to compensate for the loss of degrees of freedom and decrease in power caused by pooling samples (Shih, J.H., et. al., 2004).[1] If the author of this paper can address those disadvantages and follow suggestions in the paper referred, the different value of gene expression might be justifiable.

I would like the author to address all my concerns above appropriately, until then, in my opinion, this paper is not yet ready for indexing.

References
1. Shih JH, Michalowska AM, Dobbin K, Ye Y, et al.: Effects of pooling mRNA in microarray class comparisons.*Bioinformatics*. 2004; **20** (18): 3318-25 PubMed Abstract | Publisher Full Text

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neuroscience (any works with neurons involvement), Molecular Biology (any works related to DNA and protein).

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Version 1

Reviewer Report 16 April 2021

https://doi.org/10.5256/f1000research.29536.r76420

Widya Lestari
Department of Oral Biology, Kulliyyah of Dentistry, International Islamic University Malaysia (IIUM), Kuantan, Malaysia

This report provides new insight into dentistry. *P. gingivalis* is not only related to Periodontitis but also express in neuron cells, which may give additional knowledge to clinicians. Pathways of expression of TNF alpha and IL-1B area clearly explained. Methodology and results are well established and, we believe these findings will benefit clinicians and also researchers as well.

The methodology:
This is an in vitro study using a nerve cell culture. The experimental design has used a treatment group and a control group which, in my opinion, is good enough to observe the effect of administering anti-*P. gingivalis* antibodies on the expression of neuroinflammatory cytokines.

The results:
The results have been presented clearly. I suggest analyzing them descriptively without using statistics. This is a preliminary study, in my opinion, it has described how the expression of neuroinflammatory cytokines in nerve cell cultures after exposure to *P. gingivalis* which has been treated with anti-*P. gingivalis* antibody.

Overall, a minor revision is needed.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 25 January 2021

https://doi.org/10.5256/f1000research.29536.r76422

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Hadhimulya Asmara
Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada

The manuscript by Bachtiar *et al.* presents some potentially interesting findings on preventing the neurons from damage and death by reducing excessive release of neuroinflammatory mediators such as TNF-α and IL-1β and other molecules such as iNOS. This paper highlights the potential success of using antibody specially anti- *P. gingivalis* antibodies for suppressing factors that can damage neuronal cells can be used as a guideline for developing a *P. gingivalis* vaccine since it is one of the oral bacteria that triggers Alzheimer's disease.
Overall, the work presents an interesting idea regarding possible antibody use to decrease the release of neuroinflammatory agents and other molecules that are toxins and cause harm to the neurons. If this finding is rigorously proven, it could be a great contribution to the prevention and treatment of bacterial infection in Neurodegenerative disease. At this stage, however, there are several comments that need to be clarified in the manuscript that substantively influences the significance of the findings.

Specific comments:

1. In the paper, the authors did not mention the efficacy of other treatments such as antibiotic treatment for *P. gingivalis* infection and its effect on the release of neuroinflammatory agents. It is important to highlight the antibiotic's effect since if the antibiotic is effective enough to kill the bacteria and prevent the infection on neurons then this paper must add more reasons why this paper or the antibody approach is better than the antibiotic treatment.

2. In the figure 2. The authors used the word "morphology" of the neurons, but the authors did not explain the morphology aspect of the neurons (such as elongation axons or shape of the cell body, etc). It only described the change of the growth or number of the viable cells that was decreased by the exposure of *P. gingivalis* with or without antibody coating. It is better to describe how the exact morphological changes of the neurons as clearly shown in figure 1 before the exposure *P. gingivalis*. Even better if the authors can add the qualitative and quantitative analysis of those differences between the groups.

3. The correlation between figure 2 and figure 3. The reducing number of viable cells in figure 2 and the reducing the expression of TNF-α, IL-1β, and iNOS in figure 3 seems does not fit with the hypothesis in the paper. If the expression of TNF-α, IL-1β, and iNOS were decreased on the neurons that were exposed by coating antibody *P. gingivalis* compared to the ones without coating (figure 3) then the number of viable neurons in the figure 2c (expose with antibody) must be higher than figure 2b (without antibody) since the antibody will decrease TNF-α, IL-1β, and iNOS and it means to prevent the neuronal damage and death on figure 2b. What is the clarification or the explanation of this confusion?

4. It is necessary to state the *P* values or statistical significance of the differences between the three groups in figure 3 (with coating antibody, without coating anti, and neuron only). It is important to conclude that one group is higher or lower than the others groups based on statistical significance or *P* values.

5. As a minor comment, I think there is a typo on the first line of the second paragraph in the discussion section. In my opinion, I think the authors want to show figure 1 instead of figure 2 (as stated in that line) for describing the growth changes of neurons over time.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neuroscience (any works with neurons involvement), Molecular Biology (any works related to DNA and protein).

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 03 Mar 2021

Boy Muchlis Bachtiar, Faculty of Dentistry Universitas Indonesia, Jakarta, Indonesia

Dear Reviewer,
Thank you for the valuables suggestion in improving of the quality our manuscript.
Here we would like to respond your feedback.

Specific comments:

1. In the paper, the authors did not mention the efficacy of other treatments such as antibiotic treatment for *P. gingivalis* infection and its effect on the release of neuroinflammatory agents. It is important to highlight the antibiotic's effect since if the antibiotic is effective enough to kill the bacteria and prevent the infection on neurons then this paper must add more reasons why this paper or the antibody approach is better than the antibiotic treatment.

Author's responses:
We thank for this valuable comments. We have inserted the argumentation in the manuscript of this suggestion as follow:
P gingivalis reside in a structured community of biofilm attached to surfaces embedded in the extracellular matrix which they produce themselves and they are difficult to eradicate due to their resistance to antimicrobials and the body's defense mechanisms [10]. The passive immunization approach using polyclonal antibodies to inhibit *P gingivalis* adhesion to the periodontium tissue is a strategy to prevent biofilm formation and periodontium tissue damage which can lead to deeper tissue invasion so that *P gingivalis* can enter the systemic circulation.

1. In the figure 2. The authors used the word "morphology" of the neurons, but the authors did not explain the morphology aspect of the neurons (such as elongation axons or shape of the cell body, etc). It only described the change of the growth or number of the viable cells that was decreased by the exposure of *P. gingivalis* with or without antibody coating. It is better to describe how the exact morphological changes of the neurons as clearly shown in figure 1 before the exposure *P. gingivalis*. Even better if the authors can add the qualitative and quantitative analysis of those differences between the groups.

Author's responses:
We have deleted 'morphology' In Figure 2, it can be seen that there is a decrease in the number of neuron cells after being exposed to *P. gingivalis* bacteria.

1. The correlation between figure 2 and figure 3. The reducing number of viable cells in figure 2 and the reducing the expression of TNF-α, IL-1β, and iNOS in figure 3 seems does not fit with the hypothesis in the paper. If the expression of TNF-α, IL-1β, and iNOS were decreased on the neurons that were exposed by coating antibody *P.*

gingivalis compared to the ones without coating (figure 3) then the number of viable neurons in the figure 2c (expose with antibody) must be higher than figure 2b (without antibody) since the antibody will decrease TNF-α, IL-1β, and iNOS and it means to prevent the neuronal damage and death on figure 2b. What is the clarification or the explanation of this confusion?

Author's responses:
Qualitatively, from the microscope image (Figure 2), there was no difference between the antibody group and the non-antibody group. In our opinion, the number of cells seen on a microscope does not necessarily indicate the expression of neuroinflammation. Based on the results of the real time PCR analysis, it showed that the expression of neuroinflammation was more in the group without antibodies (Figure 3).

1. It is necessary to state the *P* values or statistical significance of the differences between the three groups in figure 3 (with coating antibody, without coating anti, and neuron only). It is important to conclude that one group is higher or lower than the others groups based on statistical significance or *P* values.

Author's responses:
We used pooled samples (we have added this information in the methods section) as there was insufficient amount of RNA from each individual replication of the experiments. But we think the value of gene expression presented here is equal to de average of 6 replications of experiment. Hence, we couldn't get a statistical significance. Some studies also use this kind of data interpretation (Shu-Dong Zhang, Timothy W. Gant, Effect of pooling samples on the efficiency of comparative studies using microarrays, Bioinformatics, Volume 21, Issue 24, 15 December 2005, Pages 4378–4383)

1. As a minor comment, I think there is a typo on the first line of the second paragraph in the discussion section. In my opinion, I think the authors want to show figure 1 instead of figure 2 (as stated in that line) for describing the growth changes of neurons over time.

Author's responses:
Thank you, we have fixed this typo.

Again thanks
With warm regards
Bachtiar EW

Competing Interests: No Competing interest

Comments on this article

Version 3

Author Response 07 May 2021

Boy Muchlis Bachtiar, Faculty of Dentistry Universitas Indonesia, Jakarta, Indonesia

Dear reviewer,

I would like to clarify the questions related mention: " The limitation to the use of 96 well plate cultures is that the number of cells in each well is very limited. Maybe for future research, using 24 well plate cultures will get a more appropriate RNA sample to be analyzed in order to overcome this problem to be able to use statistical analysis".

The reviewer's questions are:

1. Will the author change the experiments using 24 well plate in order to overcome the problem of analyzing RNA samples?
2. Will the author employ the statistical analysis as mentioned in the sentences?

The author's response:
We apologize, maybe the sentence above is confusing. What we mean is for future research the use of well 24 plate. We mean this is a discussion that might be suggested to be applied to future research so that statistical analysis can be applied.

We hope you understand what we mean and we will be happy to wait for your suggestions if there is any need to improve the manuscript.

Thanks in advance,
With warm regards,
BACHTIAR EW

Competing Interests: No competing interest were disclosed