# Prevention of streptozotocin-induced Neuro-2a cell death by C8-B4 microglia transformed with repetitive low-dose lipopolysaccharide

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Received February 2, 2021; Accepted June 29, 2021

DOI: 10.3892/mmr.2021.12328

**Abstract.** Diabetes-associated neuronal dysfunction (DAND) is one of the serious complications of diabetes, but there is currently no remedy for it. Streptozotocin [2-deoxy-2-(3-methy1-3-nitrosoureido) D-glucopyranose; STZ] is one of the most well-established diabetes inducers and has been used *in vivo* and *in vitro* DAND models. The aim of the present study was to demonstrate that C8-B4 microglia transformed by the stimulus of repetitive low-dose lipopolysaccharide (LPSx3-microglia) prevent STZ-induced Neuro-2a neuronal cell death *in vitro*. The ELISA results showed that neurotrophin-4/5 (NT-4/5) secretion was promoted in LPSx3-microglia and the cell viability assay with trypan blue staining revealed that the culture supernatant of LPSx3-microglia prevented STZ-induced neuronal cell death. In addition, reverse transcription-quantitative PCR showed that neurons treated with the culture supernatant of LPSx3-microglia promoted the gene expression of B-cell lymphoma-extra large and glucose-dependent insulinotropic polypeptide receptor. Furthermore, the inhibition of tyrosine kinase receptor B, a receptor of NT-4/5, suppressed the neuroprotective effect of LPSx3-microglia. Taken together, the present study demonstrated that LPSx3-microglia prevent STZ-induced neuronal death and that NT-4/5 may be involved in the neuroprotective mechanism of LPSx3-microglia.

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*Key words:* neuroprotection, immunomodulation, neurotrophin-4/5, tyrosine kinase receptor B, diabetes-associated, pretreatment

## Introduction

It has been elucidated that patients with diabetes are more likely to develop nervous system disorders, such as neuropathy, cognitive decline and dementia (1,2). There are 463 million patients with diabetes worldwide and half of them have been reported to develop diabetes-associated neuronal dysfunction (DAND) (1,2). However, no prophylaxis or remedy for DAND has yet been developed.

Streptozotocin [2-deoxy-2-(3-methy1-3-nitrosoureido) D-glucopyranose; STZ] is one of the most well-established diabetes inducers. *In vivo*, animals administered STZ intraventricularly have been used as experimental models for DAND (3). *In vitro*, STZ could induce neuronal damage (4-6) and is used as a model for analyzing cellular processes and potential therapies in DAND.

Microglia, the tissue-resident macrophages in the brain, are immune cells that serve a central role in innate immunity and maintain the homeostasis of the central nervous system. Microglia contribute to preventing neuronal damage by dynamically transforming their characteristics in response to various stimuli (7,8). Therefore, it is considered that inducing the transformation to neuroprotective microglia could be a solution to DAND.

Lipopolysaccharide (LPS) is a glycolipid that constitutes the outer membrane of Gram-negative bacteria and induces microglial transformation of microglia through binding to Toll-like receptor-4. It is known that a single injection of high-dose LPS induces the transformation to inflammatory microglia and neuroinflammation (9-11). By contrast, repetitive low-dose LPS stimulation could suppress neuronal dysfunction in various neurological disease models, such as Alzheimer's disease and cerebral ischemia, by inducing transformation into neuroprotective microglia both *in vivo* (12-16) and *in vitro* (17,18). Consistent with this, our previous study showed that microglia transformed by the stimulus of repetitive low-dose LPS (LPSx3-microglia) have a neuroprotective potential with high gene expression of neurotrophin-4/5 (NT-4/5) (19). NT-4/5 is a member of a family of neurotrophic factors, neurotrophins. NT-4/5 binds

to its high-affinity receptor, tyrosine receptor kinase B (TrkB) and supports neuronal survival and growth (20-23). Hence, LPSx3-microglia might have a neuroprotective effect. To analyze the effect of LPSx3-microglia on neural survival, the neuroprotective effects of LPSx3-microglia on STZ-stimulated neurons *in vitro* were evaluated (3,24).

## Materials and methods

*Microglia culture and LPS treatment*. The murine microglial cell line C8-B4 was cultured and treated with LPS, as described previously (19). Briefly, C8-B4 microglia (purchased from the American Type Culture Collection; cat. no. CRL-2540) were seeded in 12-well tissue culture plates ($2x10^5$ cells/ml) and cultured in Dulbecco's modified Eagle's medium (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C in 5% $CO_2$ (n=3). C8-B4 microglia were treated with LPS (purified LPS derived from *Pantoea agglomerans*; Macrophi, Inc.) by replacing with fresh medium containing LPS (1 ng/ml) every 24 h for a total of three times. For single treatment with LPS, cells received fresh medium without LPS for the first 48 h and then received fresh medium containing LPS once. Cell lysates and the culture supernatant were collected at 30 min and 24 h after final LPS treatment, respectively. The microglial culture supernatant (MCS) treated with LPS was filtered through a 0.22 $\mu$m polyether sulfone membrane (Sartorius AG) and frozen at -80˚C until used for culture of N2a neurons.

*Determination of NT-4/5 and prostaglandin $E_2$ ($PGE_2$) in the culture supernatant*. The culture supernatants were collected 24 h after the final LPS treatment of C8-B4 microglia. The protein levels of NT-4/5 and $PGE_2$ in LPS-treated MCS were measured using a commercial ELISA kit (Biosensis Pty. Ltd.; cat. no. BEK-2218 for NT-4/5 and Cayman Chemical Company; cat. no. 514010 for $PGE_2$) according to the manufacturer's instructions.

*Western blot analysis*. C8-B4 microglia were lysed with sodium dodecyl sulfate (SDS) sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 5% 2-mercaptoethanol at final concentration]. The total protein concentration in each sample was determined by Pierce 660 nm Protein Assay Reagent with Ionic Detergent Compatibility Reagent (Pierce; Thermo Fisher Scientific, Inc.). After boiling for 5 min, samples (5 $\mu$g protein/lane) were separated by electrophoresis on 15% polyacrylamide gels (ATTO Corporation), followed by transfer to polyvinylidene difluoride membrane (Cytiva). Following blocking with Tris-buffered saline (TBS) containing 5% bovine serum albumin (Nacalai Tesque, Inc.) at 4˚C for 1 h, the membranes were incubated with a primary antibody against extracellular signal-regulated kinase 1/2 (ERK1/2; cat. no. 4695; 1:1,000), phosphorylated (p)-ERK1/2 (Thr202/Tyr204; cat. no. 4370; 1:1,000), p-p38 (Thr180/Tyr182; cat. no. 4511; 1:1,000), p38 (cat. no. 8690; 1:1,000), CREB (cat. no. 9197; 1:1,000), p-CREB (Ser133; cat. no. 9198; 1:1,000), c-Jun (cat. no. 9165; 1:1,000), p-c-Jun (Ser73; cat. no. 3270; 1:1,000; all from Cell Signaling Technology, Inc.), RELB Proto-Oncogene NF-κB Subunit (relB; cat. no. sc-226; 1:500), or β-actin (cat. no. sc-47778; 1:500; both from Santa Cruz Biotechnology, Inc.) at 4˚C overnight. Following washing with TBS with 0.1% Tween-20, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) antibody (cat. no. 406401; 1:3,000) or HRP-conjugated anti-mouse IgG antibody (cat. no. 405306; 1:3,000; both from BioLegend, Inc.) at 4˚C for 1 h. Bound antibodies were visualized using the WesternBright ECL HRP substrate (Advansta, Inc.). Chemiluminescent signals were detected and analyzed using Amersham Imager 680 (Cytiva) according to the manufacturer's instructions. β-actin was used for normalization.

*Treatment of N2a neurons with MCS and STZ*. The murine neuroblastoma cell line Neuro-2a (N2a) was provided by the Japanese Collection of Research Bioresources Cell Bank. N2a neurons were seeded in 12-well tissue culture plates ($4x10^5$ cells/ml) and precultured in Eagle's minimal essential medium with non-essential amino acids (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C in 5% $CO_2$ for 24 h (n=3). The medium was then replaced with MCS of microglia treated with LPS (LPSx0, no treatment; LPSx1, single treatment with LPS; LPSx3, treatment with LPS three times every 24 h). After culture with LPS-treated MCS at 37˚C in 5% $CO_2$ for 24 h, 400 $\mu$M STZ (Sigma-Aldrich; Merck KGaA) was added to the culture medium and N2a neurons were cultured at 37˚C in 5% $CO_2$ for another 24 h. Finally, N2a neurons were counted after detachment by treatment with 0.25% trypsin at 37˚C in 5% $CO_2$ for 2 min. The survival rate was calculated by staining with trypan blue (Nacalai Tesque, Inc.) at room temperature for 30 sec. For TrkB inhibition, the TrkB inhibitor ANA-12 (5-30 $\mu$M; Sigma-Aldrich; Merck KGaA) was added at the same time as the replacement with LPS-treated MCS in the above procedure.

*Reverse transcription-quantitative (RT-q) PCR*. RT-qPCR was performed as described previously (19). Briefly, RNA was extracted from N2a neurons by the RNeasy Mini kit (Qiagen GmbH). cDNA was synthesized by reverse transcription using ReverTra Ace qPCR RT Master Mix (Toyobo Life Science) according to the manufacturer's instructions. qPCR assay was performed using 2 $\mu$l cDNA as template and 10 $\mu$l Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc.) on the Stratagene Mx 3005P QPCR System (Agilent Technologies, Inc.). The primers are listed in Table I. The thermocycling conditions for PCR were 95˚C for 10 min for polymerase activation, followed by 45 cycles of 95˚C for 15 sec for denaturation and 60˚C for 1 min for extension. Data were analyzed by the $2^{-\Delta\Delta Cq}$ method (25) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression.

*Statistical analysis*. A minimum sample size was determined using power analysis in a prior examination with the statistical analysis software R (26). Statistical analysis was performed using GraphPad Prism 6.0 software package (GraphPad Software, Inc.). Results are presented as mean ± standard

Table I. List of primers used for reverse transcription-quantitative PCR.

| Gene | Forward primer (5'→3') | Reverse primer (5'→3') |
|---|---|---|
| *Bcl2* | TGAGTACCTGAACCGGCATCT | GCATCCCAGCCTCCGTTAT |
| *Bcl2l1* | AACATCCCAGCTTCACATAACCCC | GCGACCCCAGTTTACTCCATCC |
| *Gapdh* | CGACTTCAACAGCAACTCCCACTCTTCC | TGGGTGGTCCAGGGTTTCTTACTCCTT |
| *Gipr* | CCGCGCTTTTCGTCAT | CCACCAAATGGCTTTGACTT |
| *Glp1r* | TCAGAGACGGTGCAGAAATG | CAGCTGACATTCACGAAGGA |
| *Glut3* | TTCTGGTCGGAATGCTCTTC | AATGTCCTCGAAAGTCCTGC |
| *Igf1r* | GTGGGGGCTCGTGTTTCTC | GATCACCGTGCAGTTTTCCA |
| *InsR* | ATGGGCTTCGGGAGAGGAT | GGATGTCCATACCAGGGCAC |
| *Ptges* | GGATGCGCTGAAACGTGGA | CAGGAATGAGTACACGAAGCC |

*Gipr*, glucose-dependent insulinotropic polypeptide receptor; *Glp1r*, glucagon-like peptide-1; *InsR*, insulin receptor; *Igf1r*, insulin-like growth factor-I receptor; *Glut3*, glucose transporter 3; *Ptges*, prostaglandin E synthase.

error of the mean. The differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or two-way ANOVA followed by Sidak's multiple comparison test. All experiments were conducted at least two times independently. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Promotion of NT-4/5 expression in LPSx3-microglia*. As our previous study revealed that NT-4/5 mRNA expression was promoted in LPSx3-microglia (19), the NT-4/5 protein level in the culture supernatant of LPSx3-microglia was confirmed by ELISA and compared to untreated control and microglia transformed by a stimulus of single low-dose LPS (LPSx1-microglia). The results showed that the protein level of NT-4/5 in the culture supernatant of LPSx3-microglia was promoted compared with the LPSx0 and LPSx1 groups (Fig. 1A).

Since NT-4/5 production was promoted by $PGE_2$ (27), the $PGE_2$ level in the culture supernatant of LPSx3-microglia was measured. As shown in Fig. 1B, LPSx3-microglia promoted the mRNA expression of prostaglandin E synthase (*Ptges*), a gene encoding microsomal $PGE_2$ synthase-1 and also increased $PGE_2$ protein levels in the culture supernatants compared with the LPSx0 group.

To assess signal transduction in LPSx3-microglia, the activation of kinases and transcription factors was measured with phosphospecific antibodies (Fig. 1C). The phosphorylation levels of ERK1/2 and p38 in the LPSx3-microglia were weaker compared with those of LPSx1-microglia, although their levels were higher compared with those of untreated controls. The phosphorylation levels of CREB were promoted in LPSx1-microglia, but suppressed in LPSx3-microglia and untreated controls. By contrast, the phosphorylation of c-Jun, which forms activator protein-1 (AP-1), was promoted in LPSx3-microglia as high as in LPSx1-microglia. RelB expression was confirmed to be suppressed in LPSx3-microglia, as reported previously in LPSx3-macrophages (28). Therefore, c-Jun may be involved in signal transduction that induces the transformation to LPSx3-microglia.

*Prevention of STZ-induced neuronal cell death by LPSx3-microglia*. To assess the effect of LPSx3-microglia on neuroprotection, the viability of N2a neurons was evaluated after STZ stimulation in the culture supernatants of untreated microglia (control MCS), culture supernatant of LPSx1-microglia (LPSx1-MCS), or culture supernatant of LPSx3-microglia (LPSx3-MCS) as shown in Fig. 2A.

Without STZ stimulation, both LPSx1-MCS and LPSx3-MCS had little effect on neuronal survival. By contrast, with STZ stimulation, LPSx1-MCS and LPSx3-MCS exhibited the opposite results in neuronal survival (Fig. 2B and C). As shown in Fig. 2B, STZ stimulation reduced the number of neurons when cultured in control MCS. By contrast, when cultured in LPSx3-MCS, no significant decrease in neuron number was observed even after STZ stimulation. Moreover, cellular viability analysis by staining with trypan blue revealed that LPSx1-MCS exacerbated the decrease in survival neuron number by STZ stimulation, whereas LPSx3-MCS suppressed STZ-induced neuronal cell death (Fig. 2C). The absolute number of surviving neurons calculated from the survival rate demonstrated that LPSx3-MCS significantly increased neuronal viability after STZ stimulation compared to control MCS and LPSx1-MCS. These results indicated that LPSx3-MCS prevents STZ-induced neuronal death.

*Promotion of B-cell lymphoma-extra large (Bcl-XL) gene expression in STZ-stimulated neurons by LPSx3-microglia*. The gene expression of *Bcl2* encoding B-cell leukemia/lymphoma-2 (Bcl-2) and *Bcl2l1* encoding Bcl-XL was analyzed in neurons after STZ stimulation in LPS-treated MCS (Fig. 3). The results showed that LPSx1-MCS suppressed *Bcl2* and *Bcl2l1* expression in neurons after STZ stimulation. By contrast, LPSx3-MCS promoted *Bcl2l1* expression in neurons after STZ stimulation. *Bcl2* gene expression also tended to be promoted by LPSx3-MCS, although no significant difference was observed. Control MCS did not affect *Bcl2* and *Bcl2l1* expression following STZ stimulation. It was also confirmed that there was no significant difference in *Bcl2* and *Bcl2l1* expression between the groups without STZ stimulation. These results indicated that Bcl-XL might be involved in the prevention mechanism of STZ-induced neuronal cell death by LPSx3-microglia.

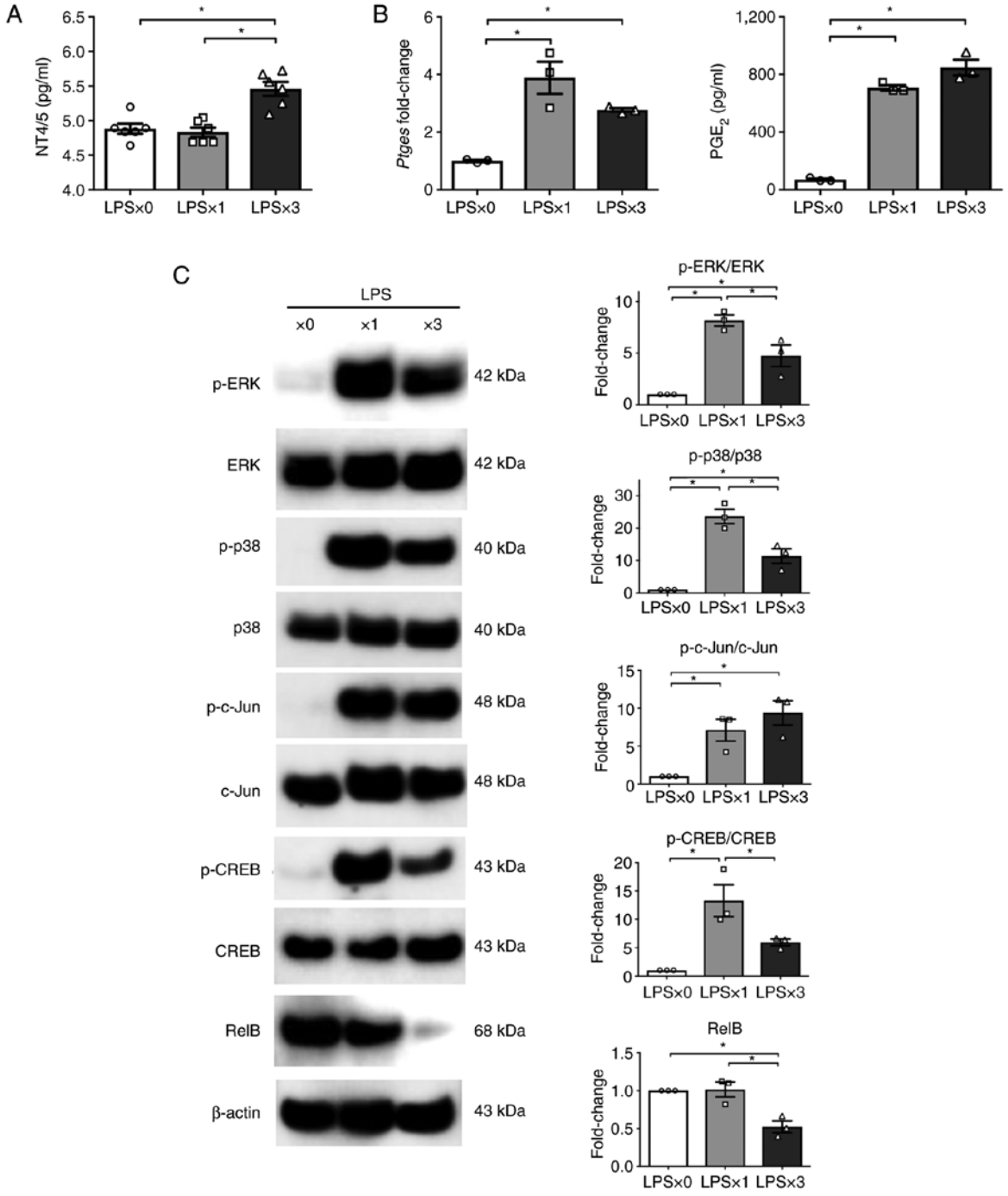


Figure 1. Promotion of NT-4/5 expression in LPSx3-microglia. (A) NT-4/5 level in the culture supernatant of C8-B4 microglia 24 h after treatment with LPS (1 ng/ml) one or three times (n=6). (B) Top, relative mRNA expression of *Ptges* of C8-B4 microglia 4 h after treatment with LPS; bottom, $PGE_2$ level in the culture supernatant of C8-B4 microglia 24 h after treatment with LPS (n=3). The relative mRNA expression was measured by reverse transcription-quantitative PCR using the $2^{-\Delta\Delta Cq}$ method. Data were normalized by GAPDH and expressed as the relative fold-change to unstimulated cells. (C) Signal transduction in microglia 30 min after treatment with LPS (n=3). Protein expression of p-ERK1/2, p-p38, p-c-Jun, p-CREB and RelB were assessed by western blotting and semi-quantified. Signal volume was normalized by β-actin and non-phosphorylated controls. Data were expressed as the relative fold-change to untreated cells. Data are presented as the mean ± SEM of each group and are representative of two independent experiments. $^*P<0.05$, one-way ANOVA with Tukey's multiple comparison test. NT-4/5, neurotrophin-4/5; LPS, lipopolysaccharide; *Ptges*, prostaglandin E synthase; $PGE_2$, prostaglandin $E_2$; p-, phosphorylated; CREB, cAMP response element-binding protein; RelB, RELB proto-oncogene NF-κB subunit; LPSx0, no treatment; LPSx1, single treatment with LPS; LPSx3, treatment with LPS three times every 24 h.

*Promotion of gene expression of glucose-dependent insulinotropic polypeptide (GIP) receptor (GIPR) in STZ-stimulated neurons by LPSx3-microglia*. GIP and glucagon-like peptide-1 (GLP-1), also called incretins, are gastrointestinal hormones that promote insulin secretion from pancreatic β-cells. Previous studies have also shown that GIP and GLP-1 have neuroprotective effects through GIPR or GLP-1 receptor (GLP1R) on neurons (29-31). Therefore, the gene expression of *Gipr* encoding GIPR and *Glp1r* encoding GLP1R was analyzed in neurons after STZ stimulation in LPSx3-MCS.

The results showed that the expression of *Gipr*, but not *Glp1r*, was significantly promoted by LPSx3-MCS in neurons

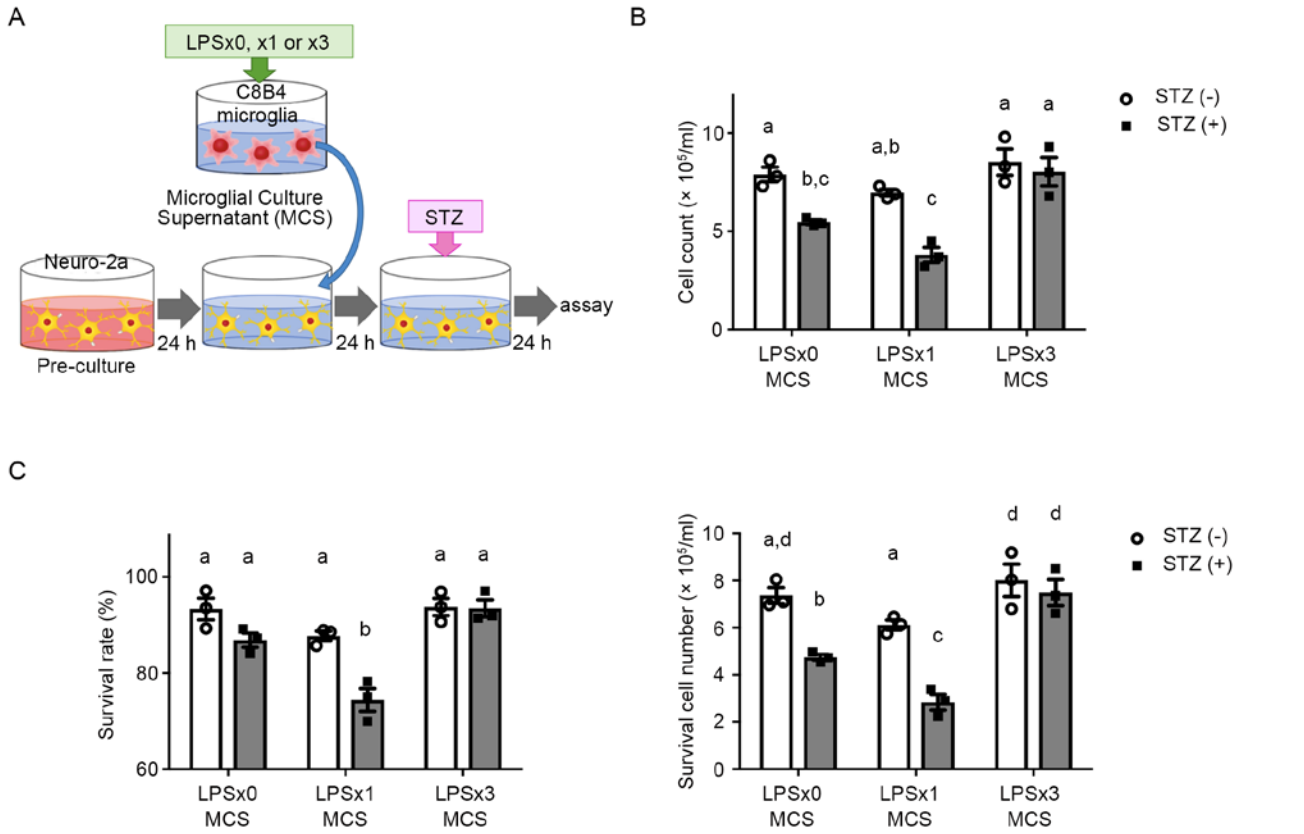


Figure 2. Prevention of STZ-induced neuronal death by the culture supernatant of LPSx3-microglia. (A) Experimental process of the evaluation of the neuroprotective effect of LPS-treated MCS in the STZ-induced neuronal damage. N2a neurons were cultured for 24 h and then the medium was replaced with MCS of microglia treated with 1 ng/ml LPS. After culture with LPS-treated MCS for 24 h, 400 $\mu$M STZ was added to the culture medium and N2a neurons were cultured for another 24 h. N2a neurons were counted and the survival rate was calculated by staining with trypan blue dye. (B) Total cell number of N2a neurons following STZ stimulation in LPS-treated MCS (n=3). N2a neurons were detached 24 h after STZ stimulation by treatment with 0.25% trypsin and the number of total cells was counted. (C) Survival rate (left) and survival cell number (right) of N2a neurons were calculated by staining with trypan blue dye (n=3). The survival rate was calculated by staining with trypan blue dye. Data are presented as the mean ± SEM of each group and are representative of two independent experiments. Two-way ANOVA followed by Sidak's multiple comparison test. $^{a-d}P<0.05$, different letters indicate statistically significant differences between groups. STZ, Streptozotocin; LPS, lipopolysaccharide; MCS, microglial culture supernatant; LPSx0, no treatment; LPSx1, single treatment with LPS; LPSx3, treatment with LPS three times every 24 h.

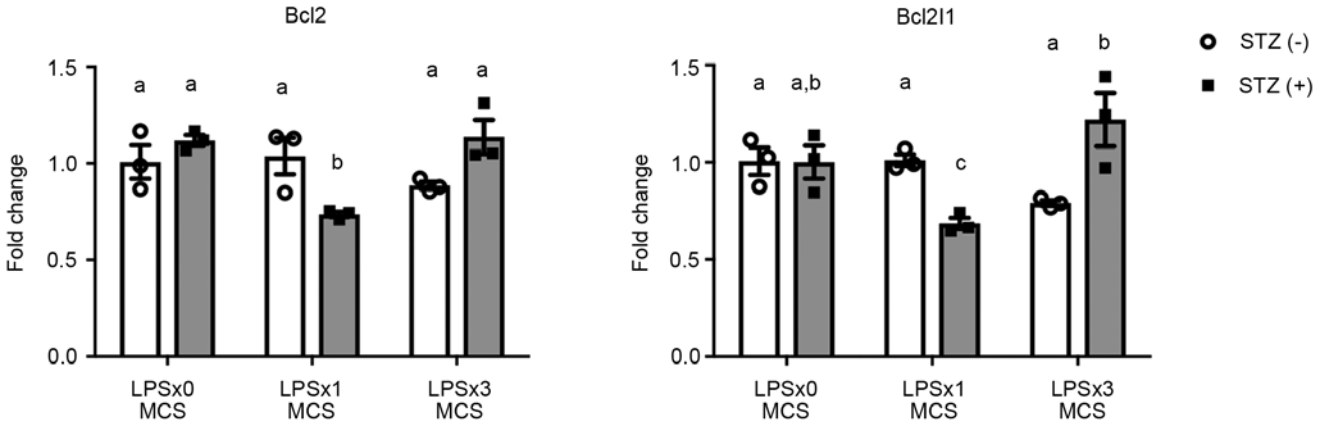


Figure 3. Promotion of Bcl-XL gene expression in STZ-stimulated neurons by the culture supernatant of LPSx3-microglia. Relative mRNA expression of *Bcl2* and *Bcl2l1* in N2a neurons was measured by reverse transcription-quantitative PCR using the $2^{-\Delta\Delta Cq}$ method 24 h after STZ stimulation in LPS-treated MCS (n=3). Data were normalized by GAPDH and expressed as the relative fold-change to unstimulated cells. Data are presented as the mean ± SEM of each group and are representative of two independent experiments. Two-way ANOVA followed by Sidak's multiple comparison test. $^{a-c}P<0.05$, different letters indicate statistically significant differences between groups. Bcl-XL, B-cell lymphoma-extra large; STZ, Streptozotocin; LPS, lipopolysaccharide; MCS, microglial culture supernatant; LPSx0, no treatment; LPSx1, single treatment with LPS; LPSx3, treatment with LPS three times every 24 h.

following STZ stimulation (Fig. 4). By contrast, control MCS and LPSx1-MCS did not affect *Gipr* and *Glp1r* expression. Therefore, GIPR may also be involved in the prevention mechanism of STZ-induced neuronal cell death by LPSx3-microglia.

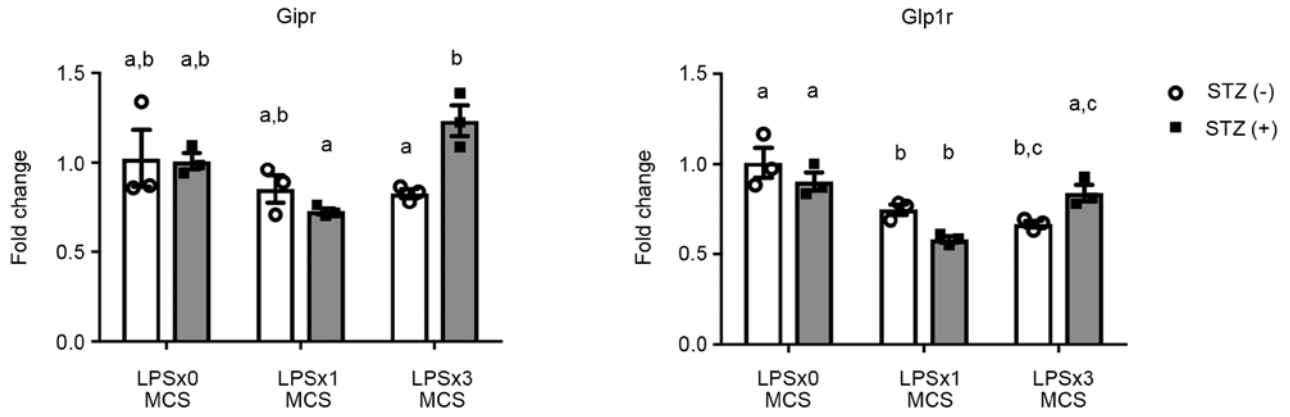


Figure 4. Promotion of *Gipr* gene expression in STZ-stimulated neurons by the culture supernatant of LPSx3-microglia. Relative mRNA expression of incretin receptor genes in N2a neurons was measured by reverse transcription-quantitative PCR using the $2^{-\Delta\Delta Cq}$ method 24 h after STZ stimulation in LPS-treated MCS (n=3). Data were normalized by GAPDH and expressed as the relative fold-change to unstimulated cells. Data are presented as the mean ± SEM of each group and are representative of two independent experiments. Two-way ANOVA followed by Sidak's multiple comparison test. $^{a-c}P<0.05$, different letters indicate statistically significant differences between groups. *Gipr*, glucose-dependent insulinotropic polypeptide receptor; STZ, Streptozotocin; LPS, lipopolysaccharide; MCS, microglial culture supernatant; LPSx0, no treatment; LPSx1, single treatment with LPS; LPSx3, treatment with LPS three times every 24 h.

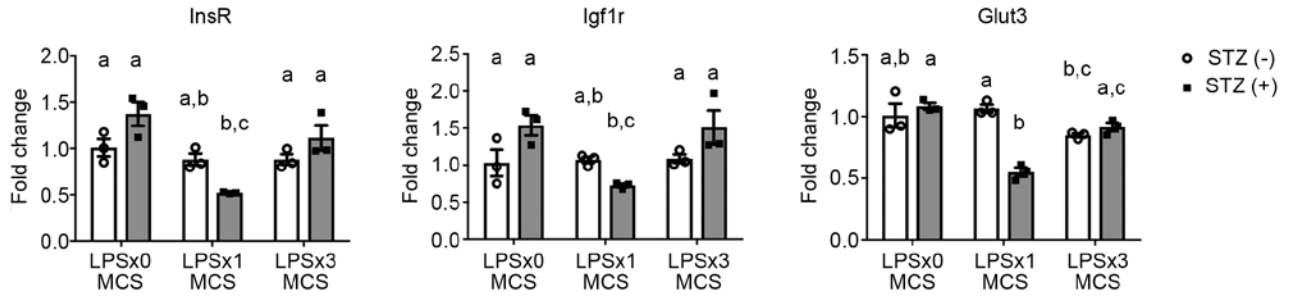


Figure 5. Unaltered glucose metabolism-regulatory gene expression in STZ-stimulated neurons by the culture supernatant of LPSx3-microglia. Relative mRNA expression of glucose metabolism regulatory genes in N2a neurons was measured by reverse transcription-quantitative PCR using the $2^{-\Delta\Delta Cq}$ method 24 h after STZ stimulation in LPS-treated MCS (n=3). Data were normalized by GAPDH and expressed as the relative fold-change to unstimulated cells. Data are presented as the mean ± SEM of each group and are representative of two independent experiments. Two-way ANOVA followed by Sidak's multiple comparison test. $^{a-c}P<0.05$, different letters indicate statistically significant differences between groups. STZ, Streptozotocin; LPS, lipopolysaccharide; MCS, microglial culture supernatant; InsR, insulin receptor; Igf1r, insulin-like growth factor-I receptor; Glut3, glucose transporter 3; LPSx0, no treatment; LPSx1, single treatment with LPS; LPSx3, treatment with LPS three times every 24 h.

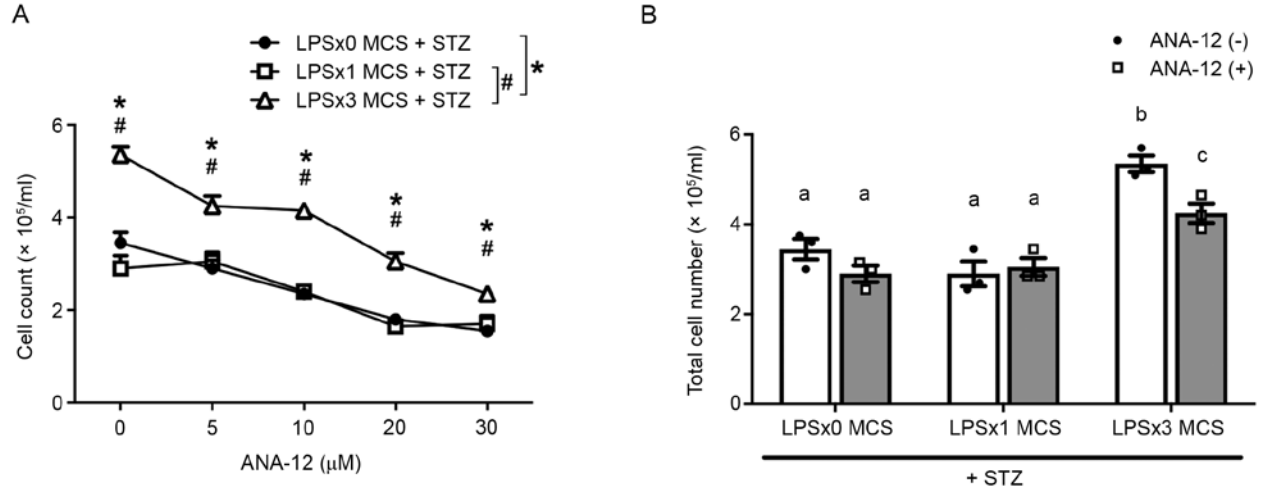


Figure 6. TrkB-dependent neuroprotective effect of LPSx3-microglia. Inhibition of the neuroprotective effect of LPSx3 microglia by ANA-12, a TrkB inhibitor. N2a neurons were cultured in LPS-treated MCS with ANA-12 at (A) 5, 10, 20 and 30 $\mu$M or (B) 5 $\mu$M followed by STZ stimulation (n=3). N2a neurons were detached 24 h after STZ stimulation by treatment with 0.25% trypsin and the number of total cells was counted. Data are presented as the mean ± SEM of each group and are representative of two independent experiments. Two-way ANOVA followed by Sidak's multiple comparison test. $^{*}P<0.05$ vs. LPSx0; $^{\#}P<0.05$ vs. LPSx1. $^{a-c}P<0.05$, different letters indicate statistically significant differences between groups. TrkB, tyrosine receptor kinase B; LPS, lipopolysaccharide; MCS, microglial culture supernatant; STZ, Streptozotocin; LPSx0, no treatment; LPSx1, single treatment with LPS; LPSx3, treatment with LPS three times every 24 h.

*Unchanging glucose metabolism regulatory gene expression in STZ-stimulated neurons by LPSx3-microglia*. The expression of the following representative genes related to glucose metabolism regulation was analyzed in neurons after STZ stimulation in LPSx3-MCS: Genes encoding insulin receptor (InsR), insulin-like growth factor-I receptor (IGF1R) and glucose transporter 3 (GLUT3).

As shown in Fig. 5, LPSx1-MCS suppressed *Glut3* expression after STZ stimulation. *InsR* gene expression also tended to be suppressed by LPSx1-MCS, although there was no significant difference. By contrast, LPSx3-MCS and control MCS did not suppress the expression of glucose metabolism regulatory genes in neurons after STZ stimulation. It was confirmed that there was almost no significant difference in the expression of the glucose metabolism regulatory genes between the groups without STZ stimulation. These results indicated that LPSx3-MCS does not affect the expression of these typical glucose metabolism genes.

*TrkB-dependent neuroprotection of LPSx3-microglia*. To confirm that NT-4/5 is involved in the neuroprotective effect of LPSx3-MCS, TrkB, a receptor of NT-4/5, was inhibited by ANA-12. N2a neurons were cultured in LPS-treated MCS with the TrkB inhibitor ANA-12 (5-30 $\mu$M) followed by STZ stimulation. The total neuron number was counted 24 h after STZ stimulation.

The results demonstrated that ANA-12 suppressed the neuroprotective effect of LPSx3-MCS in a concentration-dependent manner (Fig. 6A). By contrast, ANA-12 had little effect on the number of STZ-stimulated neurons treated with LPSx1-MCS and control MCS. As shown in Fig. 6B, this tendency was typical when stimulated with ANA-12 at a 5 $\mu$M concentration. Specifically, the number of neurons after STZ stimulation was significantly reduced by 20% by 5 $\mu$M ANA-12 treatment in LPSx3-MCS, but was not changed by 5 $\mu$M ANA-12 treatment in control MCS or LPSx1-MCS. These results indicated that the neuroprotective effect of LPSx3-MCS is TrkB dependent.

## Discussion

NT-4/5 is a neurotrophic factor with neuroprotective effects (20-23). In our previous study, NT-4/5 gene expression was promoted in LPSx3-microglia, suggesting that LPSx3-microglia have a neuroprotective potential (19).

First, it was confirmed that NT-4/5 production was promoted in LPSx3-microglia even at the protein level (Fig. 1A), which was consistent with the gene expression alterations reported in our previous study (19). Next, as NT-4/5 could be induced by $PGE_2$ (27), the $PGE_2$ level in LPSx3-microglia was analyzed. The results showed that the gene expression of *Ptges* ($PGE_2$ synthase) and $PGE_2$ production in LPSx3-microglia remained as high as that of LPSx1-microglia and were not suppressed (Fig. 1B). The promotion of $PGE_2$ in LPSx3-microglia was consistent with a previous report (32). Therefore, $PGE_2$ may be involved in NT-4/5 induction in LPSx3-microglia.

Furthermore, signal transduction analysis revealed that c-Jun phosphorylation in LPSx3-microglia was as high as in LPSx1-microglia (Fig. 1C). The transcription factor AP-1 composed of c-Jun is activated in both the cyclooxygenase 2-inducing signal involved in $PGE_2$ synthesis (33,34) and the downstream signal of $PGE_2$ (35,36). In this connection, AP-1 has also been reported to mediate the positive feedback of brain-derived neurotrophic factor (BDNF), a neurotrophin that acts as a ligand for TrkB as well as NT-4/5 (37). Hence, it can be assumed that NT-4/5 production in LPSx3-microglia may be induced by c-Jun-mediated $PGE_2$ signaling.

Given the increased NT-4/5 secretion in LPSx3-microglia, whether LPSx3-MCS prevented STZ-induced neuronal damage was then examined. The result demonstrated that STZ-induced neuronal cell death was exacerbated by LPSx1-MCS but prevented by LPSx3-MCS (Fig. 2). Incidentally, it has been reported that there is no difference in neuronal survival between culture in MCS and culture in conditioned medium (17). Conventionally, only inflammatory microglia induced by a single stimulation of high-dose LPS have been featured and there is a deep-rooted general recognition that LPS is a substance that exacerbates neuronal damage (9-11). In contrast to single LPS stimulation, the present study demonstrated that repetitive low-dose LPS stimulation prevents STZ-induced neuronal cell death *via* microglia. In fact, similar to the results of the present study, the neuroprotective effects of microglia transformed by the stimulus of repetitive low-dose LPS have been reported in models of neurological disorders other than DAND, such as Alzheimer's disease and cerebral ischemia, *in vitro* (17) and *in vivo* (12-16), supporting the results of the present study.

Next, gene expression of STZ-stimulated neurons treated with LPSx3-MCS was analyzed in order to understand the mechanism by which LPSx3-microglia prevent STZ-induced neuronal cell death.

Since NT-4/5 could suppress neuronal damage (38), the gene expression of Bcl-XL and Bcl-2 was analyzed first. The results showed that Bcl-XL gene expression in neurons after STZ stimulation was suppressed by LPSx1-MCS but was promoted by LPSx3-MCS (Fig. 3). As Bcl-XL expression is induced downstream of TrkB (39), a receptor for NT-4/5, Bcl-XL may contribute to the neuroprotective effect of LPSx3-microglia.

The gene expression of the incretin receptors GIPR and GLP1R, which have been reported to have neuroprotective effects, was analyzed. The results showed that LPSx3-MCS promoted GIPR gene expression in neurons after STZ stimulation (Fig. 4). It has been reported that GIPR activation provides a neuroprotective effect through the induction of antioxidant and neurotrophic factors (8,29-31,40). It has also been reported that TrkB activation potentiates incretin receptor signals (41). Therefore, GIPR may also be involved in the neuroprotective effect of LPSx3-microglia.

As it is known that a single LPS stimulation suppresses glucose metabolism signals (42), the expression of glucose metabolism regulatory genes was analyzed. LPSx1-MCS suppressed GLUT3 gene expression in neurons after STZ stimulation (Fig. 5). By contrast, LPSx3-MCS did not induce the suppression of the typical glucose metabolism regulatory gene expression in neurons after STZ stimulation.

Finally, to verify whether the prevention of STZ-induced neuronal cell death by LPSx3-microglia depended on NT-4/5, TrkB, the receptor for NT-4/5, was inhibited. The result demonstrated that the effect of LPSx3-microglia on preventing STZ-induced neuronal cell death is TrkB

dependent (Fig. 6). As BDNF, another ligand for TrkB, is not induced in LPSx3-microglia (19), it was hypothesized that the TrkB-dependent prevention of neuronal cell death by LPSx3-microglia may be mediated by NT-4/5.

The present study did not exclude the effects of neuroprotective molecules other than NT-4/5, because LPSx3-microglia also promote the expression of neuroprotective genes other than NT-4/5 (19). However, considering the current situation that the neuroprotective mechanism by LPSx3-microglia is unclear, it is an important achievement that at least NT-4/5 is likely to be a key factor in the neuroprotective mechanism of LPSx3-microglia. Although the number of repetitions of the experiment was slightly small, almost no variation between the experiments was observed, indicating the validity of these results. To elucidate the neuroprotective mechanism of LPSx3-microglia using the present study as a foothold, further research is needed, including protein expression analysis of Bcl-XL and GIPR, gene knockdown and neutralization experiments.

The current treatment for DAND is glucose control and lifestyle modifications for mild cases. In severe cases, pain remedies and blood flow improvers are prescribed, both of which are symptomatic treatments (43). In addition, an aldose reductase inhibitor for suppressing sorbitol production, which is one of the causative substances of DAND, may also be prescribed, but the problem is that it has strong side effects (44). Therefore, treatment of DAND is very difficult due to the lack of radical pathogenetic therapy. New treatments using polyphenols are expected, but they have not yet been clinically applied to the research stage (45). Given this situation, further therapeutic research is needed to resolve DAND. The present study found the neuroprotective effect of microglia transformed by the stimulus of repetitive low-dose LPS as a novel potential treatment for DAND. Considering the various characteristics of microglia as immune cells, such as phagocytic activity, inflammatory regulation and tissue repair (46), microglia transformed by the stimulus of repetitive low-dose LPS may be expected to have a multitarget therapeutic effect.

In conclusion, the results demonstrated that the culture supernatants of LPSx3-microglia prevent STZ-induced neuronal cell death. It also found that the promotion of gene expression of Bcl-XL and incretin receptor GIPR may be involved in the neuroprotective mechanism of LPSx3-microglia. Moreover, the TrkB inhibitor suppressed the neuroprotective effect of LPSx3-microglia, suggesting that NT-4/5 is involved in this mechanism. These findings may help understand the protective role of microglia in DAND, where these cells are usually regarded as inflammatory or harmful.

**Acknowledgements**

Not applicable.

**Funding**

The present study was funded by the Control of Innate Immunity, Collaborative Innovation Partnership and supported by a grant from the Cross-ministerial Strategic Innovation Promotion Program (grant no. SIP 14533073) from the Council for Science from Technology and Innovation in the Cabinet Office of the Japanese Government and the National Agriculture and Food Research Organization.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

HM, HI, CK and GIS conceptualized the study and coordinated the experiments. HM, KY and MY performed the experiments. HM, KY and HI confirm the authenticity of all the raw data. HM performed data curation and formal analysis. HI, CK and GIS acquired funding and administrated the project. HM wrote the manuscript as supervised by HI, CK and GIS, with contributions from all authors. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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