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ORIGINAL ARTICLE

Conditioned-medium of stem cells from human exfoliated deciduous teeth prevent apoptosis of neural progenitors



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Abstract *Purpose:* This study aimed to evaluate the neuroprotective ability of the conditioned medium of stem cells from human exfoliated deciduous teeth (CM-SHED) to prevent glutamate-induced apoptosis of neural progenitors.

Materials and methods: Neural progenitors were isolated from two-day-old rat brains, and the conditioned medium was obtained from a mesenchymal stem cell SHED. Four groups were examined: neural progenitor cells cultured in neurobasal medium with (N+) and without (N-) glutamate and glycine, and neural progenitor cells cultured in CM-SHED with (K+) and without (K-) glutamate and glycine.

Results: The expression of GABA A1 receptor (*GABAAR1*) messenger RNA (mRNA) in neural progenitor measured by real-time quantitative PCR. GABA contents were measured by enzyme-linked immunosorbent assay, whereas the apoptosis markers caspase-3 and 7-aminoactinomycin D were analysed with a Muse® cell analyzer. The viability of neural progenitor cells in the K+ group (78.05 %) was higher than the control group N- (73.22 %) and lower in the N+ group (68.90 %) than in the control group. The K+ group showed the highest GABA con-

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tent, which significantly differed from that in the other groups, whereas the lowest content was observed in the N + group. The expression level of *GABAARI* mRNA in the K + group was the highest compared to that in the other groups. CM-SHED potently protected the neural progenitors from apoptosis.

Conclusions: CM-SHED may effectively prevent glutamate-induced apoptosis of neural progenitors.

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1. Introduction

Neural death is a major symptom in neurodegenerative diseases such as Alzheimer's, Parkinson's, multiple sclerosis, and Huntington's disease. Apoptosis results from a trigger that activates a signal transduction pathway leading to cell death. Some triggers for apoptosis in chronic neurodegenerative diseases include excitotoxicity mediated by increased extracellular glutamate levels, increased formation of free radicals, induced DNA damage, increased expression of genes encoding the p53 protein, and damage to the plasma membrane (Kermer et al., 2004, Taylor et al., 2008). Excitotoxicity is an essential component of the pathogenesis of brain damage during the perinatal period. This process is initiated by elevated extracellular glutamate levels, which lead to hyperactivity of the glutamate receptors, increasing calcium (Ca^{2+}) influx and ultimately resulting in apoptosis (Allen et al., 2004, Almeida et al., 2005). Glutamate can be converted into gamma-aminobutyric acid (GABA) by the enzyme glutamic acid decarboxylase (GAD). Thus, GAD reduces extracellular glutamate levels. In mature neurons, GABA is a major inhibitory neurotransmitter of the central nervous system, whereas in neural progenitors, GABA can cause chlorine (Cl^-) efflux, resulting in depolarization which leads to neural excitation (Brooks-Kayal and Russek 2012, Egusa et al., 2012). To perform its function, GABA must bind its receptor. The GABA A1 receptor (GABAAR1) is the most common GABA receptor in the brain, which may inhibit apoptosis when activated. Caspase-3 is expressed as its inactive form pro-caspase-3, which has a nitrosylated cysteine residue in its catalytic site. During apoptosis, denitrosylation of this cysteine residue converts pro-caspase-3 into its active form. Activation of GABAAR1 may inhibit this denitrosylation, thereby maintaining caspase-3 in its inactive form and inhibiting apoptosis (Esmaeili et al., 2014). Glutamate receptor hyperactivity causes an increase in Ca^{2+} influx into cells, which triggers apoptosis (Finucane et al., 1999, Nechushtan et al., 1999, Pawlowski and Kraft 2000, Banke and Traynelis 2003, S Smaili et al., 2011).

One of the treatment options under development for neurodegenerative diseases is using stem cells. Previous studies have reported that human adipose derived stem cells (hADSCs) are capable of differentiating directly into dopaminergic neurons (Spitzer 2006). Stem cells from human exfoliated deciduous teeth (SHED) are adult stem cells derived from the deciduous dental pulp tissue. Stem cell lines were first isolated from human deciduous teeth by tooth exfoliation. (Wei et al., 2012). SHED have the following advantages: the retrieval process is not invasive, and cell availability is unlimited (Miura

et al., 2003, Wei et al., 2012, Khademizadeh et al., 2019). SHED also possess neuroregenerative and neuroprotective capabilities owing to the secretion of their metabolites into their conditioned medium (CM) (Kerkis and Caplan 2012, Inoue et al., 2013). One such metabolite is GAD. In this study, we assessed the neuroprotective potency of the conditioned medium of stem cells from human exfoliated deciduous. This study provides a scientific basis for the use of CM-SHED to treat neurodegenerative diseases, which has not been previously reported.

2. Materials and methods

2.1. Cultures of neural progenitors and CM-SHED

Neural progenitors were obtained from the brain of two-days-old Sprague–Dawley rats (*Rattus norvegicus*). SHED were isolated and characterized as previously reported (Inoue et al., 2013). CM-SHED was produced following a previously published method (Zainuri et al., 2018). Briefly, SHED culture which had reached 90 % confluence, was added with osteocyte induction medium (Stempro osteogenesis differentiation kit Gibco A10072-01), adipocytes (Stempro adipogenesis differentiation kit Gibco A10070-01) and chondrocytes (Stempro Chondrogenesis differentiation kit Gibco A10071-01).

Neural progenitors were isolated as described in previous studies (Lee et al., 2009, Zainuri et al., 2018). The materials used for neural progenitor cell isolation were as follows: trypsin-ethylenediaminetetraacetic acid (EDTA) (T3924; Sigma Aldrich, Saint Louis, USA), phosphate-buffered saline (PBS) (10X), pH 7.4 (70011069; Gibco, Carlsbad, USA), Hanks' balanced salt solution (HBSS) (14175-095; Gibco), glucose (G8270; Sigma Aldrich, Saint Louis, USA), Ficoll-PaqueTM plus (17-1440-02; GE Healthcare, Uppsala, Sweden), sodium chloride (NaCl) (746398; Sigma Aldrich, Saint Louis, USA), potassium chloride (KCl) (P9541; Sigma Aldrich, Saint Louis, USA), sodium dihydrogen phosphate (NaH_2PO_4) (S3139; Sigma Aldrich, Saint Louis, USA), potassium dihydrogen phosphate (KH_2PO_4) (P5655; Sigma Aldrich, Saint Louis, USA), sodium bicarbonate (NaHCO_3) (S7277; Sigma Aldrich, Saint Louis, USA), EDTA (E6758; Sigma Aldrich, Saint Louis, USA), toluidine blue (198161; Sigma Aldrich, Saint Louis, USA), poly-D-lysine (P6407; Sigma Aldrich, Saint Louis, USA), neurobasal medium (12349015; Gibco, Carlsbad, USA), B27 supplement (17504044; Gibco, Carlsbad, USA), fetal bovine serum (FBS) (F4135; Sigma Aldrich, Saint Louis, USA), antibiotic–antimycotic (100X) (15240062; Gibco, Carlsbad, USA), and GlutaMAX supplement (35050; Gibco, Carlsbad, USA).

Table 1 Flow cytometry characterization of stem cells from human exfoliated deciduous teeth (SHED).

Marker	Passage (%)					
	P2	P3	P4	P5	P7	P8
CD90 +	99.7	98.7	99.9	99.9	97.4	100.0
CD73 +	99.3	98.6	99.9	99.9	97.4	100.0
CD105 +	70.3	86.6	99.7	99.8	95.1	60.9
CD90 +, CD73 +	99.2	98.6	99.1	99.5	97.4	100.0
CD90 +, CD73 +, CD105 +	66.2	86.5	96.6	99.2	85.8	61.0

2.2. Identification of neural cell phenotype by flow cytometry

Flow cytometry characterization of SHED was carried out (**Table 1**). The polysialic acid neural cell adhesion molecule (PSA-NCAM) + and A2B5-PSA neural progenitor markers were characterized using A2B5 (-) Rat (APC) (130093582; Miltenyi Biotec Inc., Auburn, USA), PSA-NCAM(+) Rat (PE) antibody (130-120-437; Miltenyi Biotec Inc.), isotype phycoerythrin (PE) conjugated immunoglobulin M (IgM) (130099127; Miltenyi Biotec Inc.), and isotype allophycocyanin (APC) conjugated IgM (130102673; Miltenyi Biotec Inc.). On the first day after isolation, the neural progenitors were divided into the following four groups: neural progenitors cultured only in neurobasal medium (N- group), neural progenitors cultured in neurobasal medium-plus glutamate (10 μ M) and glycine (1 μ M) (N + group), progenitors cultured in neurobasal medium with CM-SHED without glutamate and glycine (K- group), and neural progenitors cultured in neurobasal medium with CM-SHED plus glutamate (10 μ M) and glycine (1 μ M) (K + group). After 24 h, GABA, GABAAR1 and GABAAR1 mRNA levels in neural progenitors were measured.

Neural progenitors were characterized using antibodies against A2B5, the marker for immature glial-committed precursors (130093582; Miltenyi Biotec, Inc., Bergisch Gladbach, Germany), and against PSA-NCAM (130-120-437; Miltenyi Biotec, Inc.).

2.3. mRNA expression analysis by real-time quantitative polymerase chain reaction (RT-qPCR)

The relative expression of mRNA encoding *GABAAR1* in the neural progenitors was determined using the $2^{-\Delta\Delta Ct}$ method. The housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control. *GABAAR1* mRNA expression was determined using Genezel™ (GZR100; Geneaid Biotech, Taipei, Taiwan). The following primers were used: *GABAAR1*, forward primer GAGGG-TATCGTGGGATG and reverse primer GGCTTGACTT CTTTCGGTTC; *GADPH*, forward primer AAGATGGT-GAAGGTCGGTGT and reverse primer TGGAAGATGGT-GATGGGTTT. A One-Step SYBR® PrimeScript™ RT-PCR Kit II (RR086A v.0709; Takara Bio, Shiga, Japan) was used for amplification. Apoptosis was analysed using a Muse® Caspase-3/7 Kit (MCH100108, Millipore, Billerica, MA, USA). Treated neural cells were stained with the Muse®

Caspase-3/7 Kit and analyzed with the Muse® Cell Analyzer (EMD Millipore, Billerica, MA, USA).

Glutamate (1017911000; Merck Millipore, Darmstadt, Germany) was used to induce neural progenitor damage. Glycine was purchased from Merck Millipore (1042010100).

Cell viability was analysed using trypan blue (T8154; Sigma-Aldrich., St. Louis, MO, USA). The GABA concentration was determined using the enzyme-linked immunosorbent assay (ELISA) kit for GABA (MBS269152; MyBioSource, Inc., San Diego, CA, USA). The GABAAR1 content was measured using an ELISA kit for GABAAR1 (MBS9342109; MyBioSource, Inc.).

2.4. Ethics statement

This study has been approved by the National Board of Health Research and Development Jakarta, Indonesia (approval number LB.02.01 / 5.2 / KE.055).

2.5. Statistical analysis

If the data distribution was normal, analysis of variance (ANOVA) was carried out to determine the differences between more than two groups. In case of abnormal data distribution, the Kruskal-Wallis test followed by the Mann-Whitney test was performed.

3. Results

The neural progenitors' biomarkers PSA-NCAM + and A2B5- were characterized using flow cytometry. Among the five fractions, only fractions two, four, and five had a population of PSA-NCAM + and A2B5- cells above 80 %. (**Fig. 1**).

This study showed that the groups of neural progenitors cultured with CM-SHED (K + and K-) had higher viability than those cultured without CM-SHED (N + and N-). After treatment, the percentage of live neural progenitors in the N + group was lower than that in the other groups. The effect of CM-SHED on the viability of neural progenitors is demonstrated in **Table 2**. While the viability of neuron progenitors in the K + group (78.05 %) was higher than that in the N- control group (73.22 %), the N + group had a lower viability (68.90 %).

Among all the of experiments, the highest and lowest level of GABA was observed in the K + and N + group, respectively (**Fig. 2**). There was a significant difference ($p < 0.05$) in the GABA content between the K + group and the other groups.

GABAAR1 content was observed to be significantly higher in the N + group than the other groups ($p < 0.05$) (**Fig. 3**). The expression of *GABAAR1* mRNA in the K + group was higher than that in the N- group ($p < 0.05$), whereas in the N + and K- groups, the levels were lower than the N- group ($p < 0.05$) (**Fig. 4**).

4. Discussion

In the present study, we found that neural progenitors cultured with CM-SHED were more viable than those cultured without CM-SHED were. This is presumably due to the role of CM-

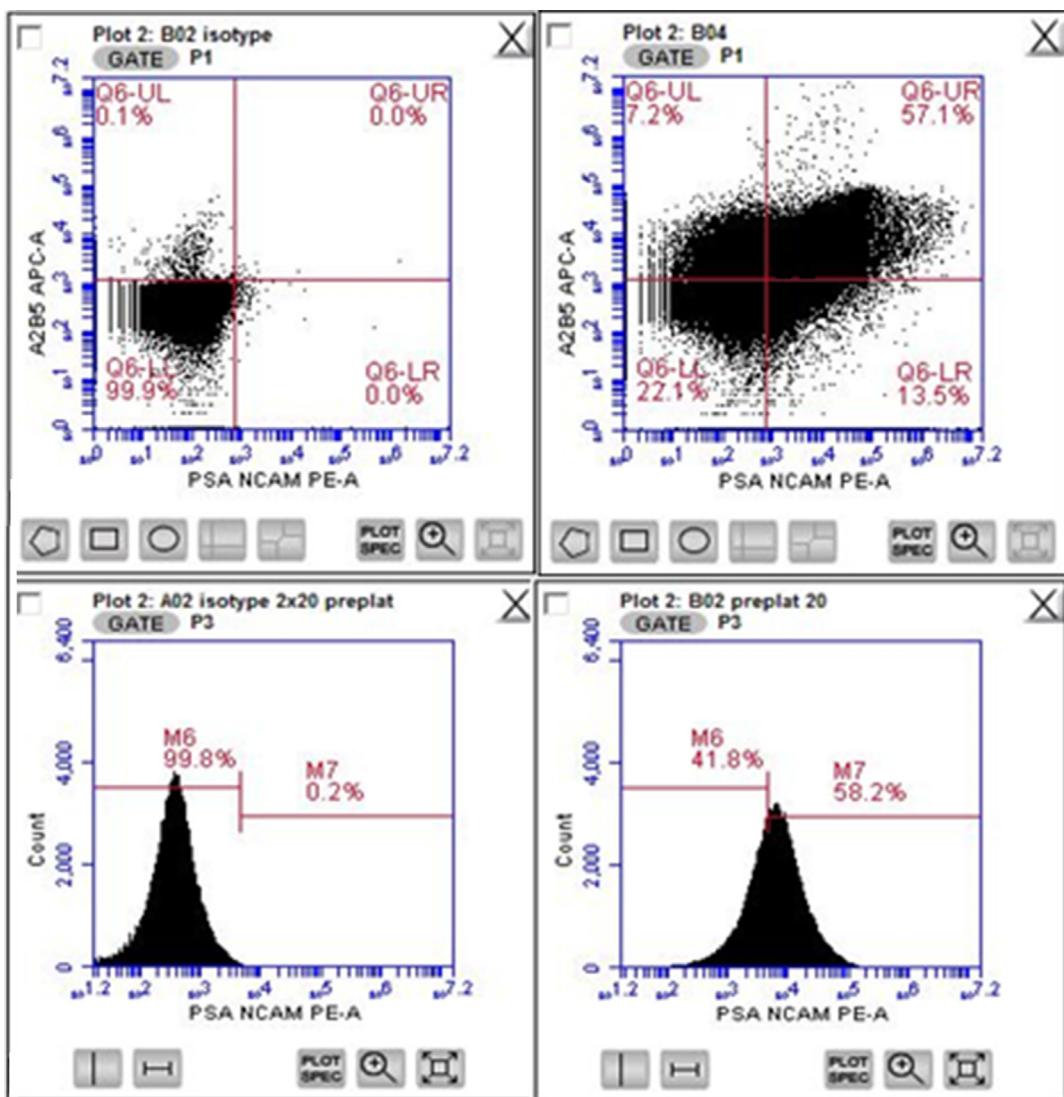


Fig. 1 Flow cytometry identification of neuron progenitor phenotypes with polysialic acid neural cell adhesion molecule (PSA-NCAM)⁺A2B5⁻ markers. Purity was 95.86 %.

Table 2 Effect of conditioned medium of stem cells from human exfoliated deciduous teeth (CM-SHED) on the viability of neural progenitors.

Neural Progenitor Condition (%)	N-	N +	K-	K +
Caspase-3 (-) and 7AAD (-) / Live	73.22	68.9	78.58	78.05
Caspase-3 (+) and 7AAD (-) / Early Apoptosis	0.46	0.36	0.77	0.35
Caspase-3 (+) and 7AAD (+) / Late Apoptosis	9.36	11.07	5.07	6.14
Caspase-3 (-) and 7AAD (+) / Dead	16.96	19.67	15.58	15.45

SHED in protecting neural progenitors from damage induced by glutamate. This result is consistent with a previous study, which reported CM-SHED to suppress neuron death by glutamate induction and by several factors in CM-SHED that are involved in various neuroregenerative mechanisms, such as neuroprotection, axon extension, neurotransmission, inflammatory suppression, and microglia regulation (Lee et al., 2009). Inoue et al. reported that CM-SHED could increase the migration and differentiation of endogenous neural pro-

genitor cells, thereby reducing ischemic brain damage (Inoue et al., 2013). Other studies have reported that the addition of CM-SHED might inhibit apoptosis, reduce tissue loss in the brain, and improve neurological function in mice with ischemic hypoxia (Mita et al., 2015, Wang et al., 2018). Another study reported that the upregulation of the phosphoinositide-3-kinase (PI3K)/Akt pathway prevents neuronal apoptosis via regulation of *BCL2* and *BAX* expression (Yamagata et al., 2013). Yamaza et al. reported the ability of SHED to express

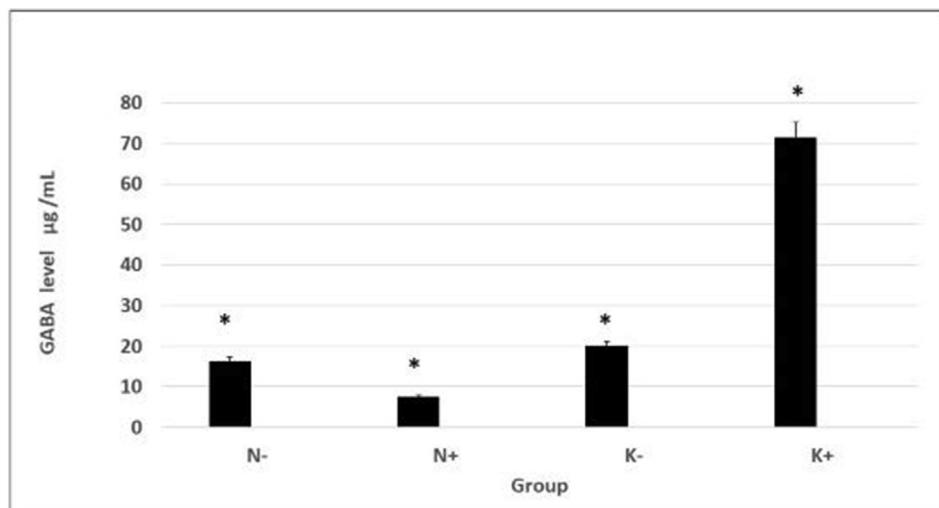


Fig. 2 Effect of conditioned medium of stem cells from human exfoliated deciduous teeth (CM-SHED) with or without the addition of glutamate and glycine on gamma-aminobutyric acid (GABA) levels in neural cell culture. *Significant difference, $p < 0.05$.

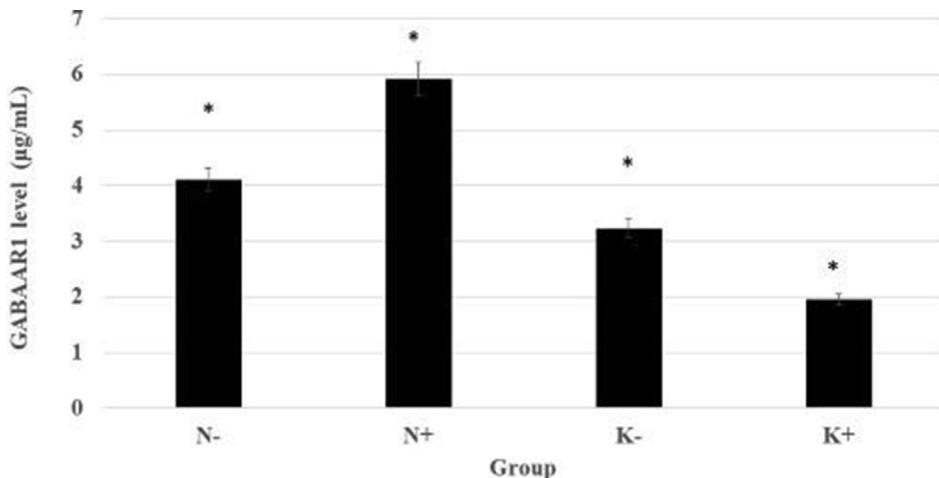


Fig. 3 Effect of conditioned medium of stem cells from human exfoliated deciduous teeth (CM-SHED) with or without the addition of glutamate and glycine on the level GABA A1 receptor (GABAARI) protein in neural cell culture. *Significant difference, $p < 0.05$.

the transforming growth factor- β 1 (TGF- β 1) and TGF- β 2 receptor, platelet-derived growth factor (PDGF) receptor, extracellular signal-regulated kinases (ERK), phosphorylated ERK, Akt, and phosphorylated Akt (Yamaza et al., 2010).

This study showed no significant difference regarding GABA content between the K- and N- groups, which were not subjected to glutamate induction. This result might be caused by normal glutamate content in the CM. The addition of glutamate and CM-SHED containing GAD catalyzes the glutamate-to-GABA conversion, resulting in high GABA content. Miura et al. found that SHED could express the GAD enzyme (Miura et al., 2003). Another study reported the ability of SHED to express neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) (Esmaili et al., 2014). One previous study reported that BDNF might enhance the release of GABA via the mitogen-activated protein kinase (MAPK) signaling pathway and cyclic adenosine

monophosphate-response element binding protein (CREB) transcription factors (Yamagata et al., 2013).

The expression of *GABAARI* mRNA was highest in the K+ group compared to that in the other groups. This is probably due to the GABA-mediated activation of the MAPK pathway and phosphorylation of CREB at the serine 133 residue. CREB transcription factors are active when bound to other CREB family members, forming a heterodimer structure. Thereafter, they bind to the cAMP response element (CRE) in the promoter region of *GABAARI*, which has a TGACGTCA motif, resulting in *GABAARI* mRNA transcription. It has been proven that the administration of muscimol, a GABAARI agonist, causes a subunit change from γ 2 to non- γ 2 in the GABAARI synaptic region, resulting in decreased GABAergic synapse strength (Yamaza et al., 2010). In the present study, the lowest GABA content was found in the N+ group, presumably due to the binding of GABA to

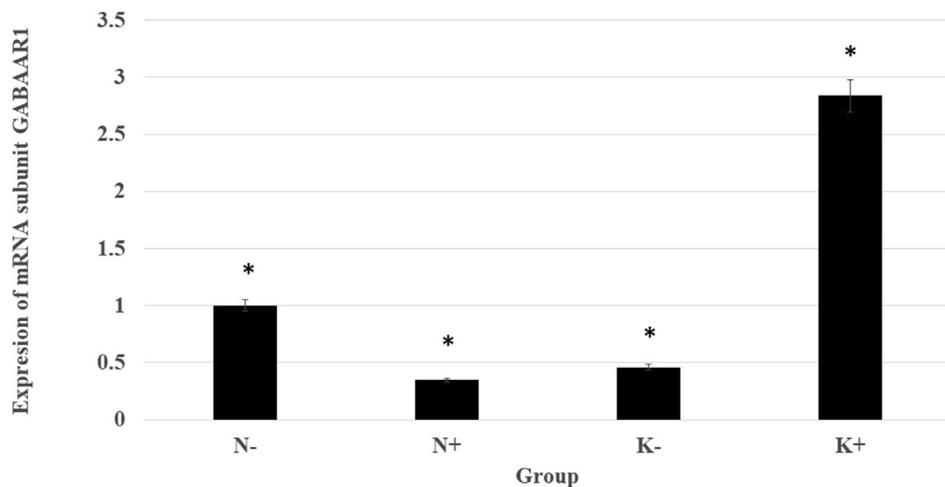


Fig. 4 Effect of conditioned medium of stem cells from human exfoliated deciduous teeth (CM-SHED) with or without the addition of glutamate and glycine on expression of mRNA subunit of GABA A1 receptor (GABAAR1) in neural cell culture. *Significant difference, $p < 0.05$.

GABAAR1. GABAAR1 content was highest in the N + group. Another possibility for the reduced GABA content in the N + group is the negative feedback from GABAAR1. It has been reported that GABAAR1 activation lowers the GAD65 content via the BDNF/ tropomyosin receptor kinase B (TrkB) signaling pathway. Decreased levels of GAD65 cause a reduction in GABA (Obrietan et al., 2002). The high concentration of GABAAR1 in the N + group was also thought to be due to BDNF, which increases GABAAR1 protein expression on the cell surface via the TrkB, protein kinase C (PKC), and PI3K pathways. In each pathway, BDNF exerts positive feedback on GABAAR1 (Matsuzaki et al., 1999). The present study reports a decreased GABAAR1 mRNA expression in the N + group compared to that in the control group. This decrease could be either because the protein was not required, or may be induced by BDNF that decreases the transcription of GABAAR1 mRNA via the Janus kinase/ signal transducer and activator of transcription (JAK/STAT) pathway. BDNF activates the phosphorylation process in STAT3 signaling, thereby initiating the formation of inducible cAMP early repressor (ICER), which binds to inactive, phosphorylated CREB (pCREB), thus inhibiting the binding of pCREB to CRE in the promoter of GABAAR1 (Matsuzaki et al., 1999, Lund et al., 2008, Porcher et al., 2011).

There was no significant difference in the content of GABAAR1 protein between the N- and K- groups, because GABA content was similar in both the groups. Khalilov et al. reported a dynamic change in progenitor neuron GABAAR1 regulation before and after the peak of giant depolarizing potentials (GDPs) (Khalilov et al., 2015). The increase in GABA reported in the K + group may be due to postoperative GDP inhibition of GABAAR1 activity, compensating for excitatory glutamate activity and resulting in neuroprotection.

In the present study, the suspected postoperative GDP inhibition of GABAAR1 activity in the N + group could not overcome the excitation from glutamate induction, leading to apoptosis. In the K + group, where there was an increase in GABA due to CM-SHED, the suspected postoperative GDP inhibition of GABAAR1 activity in the N + group could not overcome the excitation from glutamate induction, leading

to apoptosis. can compensate for the excitatory activity by glutamate resulting in neuroprotection. The primary limitation of this study is that we did not identify the composition of CM-SHED. Hence, further studies are warranted to identify the proteins in CM-SHED that play a role in neuroprotection. Further, a similar molecular docking study to that reported by Asadollahi et al. (Asadollahi et al., 2019) might be needed to analyse the interaction between GABAAR1 and GDPs.

5. Conclusion

CM-SHED may effectively prevent glutamate-induced apoptosis of neural progenitors, as indicated by the expression level of GABAAR1 mRNA being highest in the K + group. This study reveals that stem cells from human exfoliated deciduous teeth are a resource, which may be used for regenerative therapy in the medical-dental field.

Ethical statement

This study 'Conditioned-Medium of Stem Cells from Human Exfoliated Deciduous Teeth Prevent Apoptosis of Neural Progenitors' obtained ethical approval from National Board of Health Research and Development with the ethical approval number LB.02.01 / 5.2 / KE.055.

CRediT authorship contribution statement

Masagus Zainuri: Data curation, Formal analysis, Writing – original draft. **Jan Purba:** Data curation, Formal analysis, Investigation. **Sri WA Jusman:** Conceptualization, Supervision, Resources, Validation. **Endang W Bachtiar:** Project administration, Visualization, Writing – review & editing.

Declaration of Competing Interest

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

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