The Appropriate Dose of Curcumin Induces The Cell Differentiation of Cortical Neural Stem Cells (NSCs)

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ABSTRACT
Research on herbal curcumin is still developing, one of which is its role in stimulating the proliferation and differentiation of neural stem cells (NSC). The study used curcumin to stimulate neural stem cell proliferation to obtain the optimal dose for the proliferation process. We used the concentration of curcumin in this study was 0.1 µM, 0.5 µM, one µM, and two µM. The positive control used Synthetic growth factors are Basic Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), and heparin. Meanwhile, Dimethyl Sulfoxide (DMSO) solution served as a negative control. Analysis of NSC proliferation ability using the water-soluble tetrazolium salt test (WST-1). As a marker for neural stem cell proliferation, the expression of the microtubule associated protein 2 (MAP-2), Nestin gene, and the sex-determining Y-box 2 (SOX2) gene by RT-PCR. Cell morphology analysis showed that cells proliferated optimally at a dose of 0.5 µM curcumin. One-way ANOVA analysis and the results of the Tukey post hoc test on the WST-1 test showed that a curcumin concentration of 0.5 µM was optimal to stimulate cell proliferation. The highest Sox2, MAP-2, and Nestin gene expression test results occurred with 0.5 µM curcumin. The results showed that a concentration of 0.5 µM of curcumin could stimulate NSC cell proliferation as well as stimulation by synthetic growth factors.

Keywords: *Gene expression; MAP gene, Nestin gene; qPCR; SOX-2 gene*

1. INTRODUCTION

Cortical Neural Stem Cells (NSCs) can renew nerve cells, reducing the occurrence of abnormalities in degenerative diseases. [1][2] Researchers are refining protocols to stimulate the differentiation of neural stem cells into specific nervous system cell types for therapeutic and disease-modeling purposes.[3] Intrinsic genes, epigenetics, and extrinsic genes play a role in stimulating regeneration through stem cells. Several studies have shown that neural stem cells have limitations in neurogenesis.[4] NSC cell culture requires growth factors to stimulate the process of cell proliferation and differentiation.[5]

Commercial growth factors commonly used in stem cell research are relatively expensive.[6] Curcumin is an alternative natural growth factor because it stimulates cell proliferation and differentiation. [7][8] The role of Curcumin in NSCs and hippocampal neurogenesis has been studied using mice as an animal model, showing the results of a significant increase in memory function with an increase in the number of new NSCs and new neurons. [9] Pre-research results show that a curcumin dose of 0.2 mg/kg can activate NSC proliferation, increase neurogenesis, and improve cognitive impairment in rat animal models. [10]

During the proliferation and differentiation process, embryonic stem cells and adult NSCs will express high levels of the SOX2 gene.[11] SOX2 will stimulate cell differentiation and renewal of adult brain capacity.[1] NESTIN is a marker for neural stem cells or progenitor cells during the development of the central nervous system (CNS) and is a filamentous protein that forms nerve cells. During embryogenesis, most cells show positive NESTIN expression in cell proliferation. MAP-2 is very good at distinguishing NSC stem cells from adult neurons.[7][12] The MAP-2 gene plays a role in microtubule assembly and the process of neurogenesis. [13] (Mohammad et al., 2016).

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Curcumin has the active ingredient diferuloyl-methane, which has many biological effects, including anti-inflammatory, antioxidant effects, and cell apoptosis. [14][9] Recent research using fetal rat brains revealed that curcumin increases neurogenesis. However, there has been no IN VIVO study investigating whether Curcumin affects the differentiation and proliferation of spinal cord NS Cs (SC-NSPCs) in spinal cord injury (SCI) and hippocampus.[15] Therefore, this study aims to analyze the appropriate concentration of Curcumin > 0.2 μM using cortical NSC mice that can optimally stimulate neuronal cell proliferation and differentiation. The Water Soluble Tetrazolium Salt-1 (WST-1) is used to analyze the morphology and the number of proliferating cells. The expression of NESTIN, SOX2, and MAP-2 genes, as markers of nerve cell proliferation, are analyzed by the q-PCR method.

2. MATERIAL AND METHOD

2.1 Material

The research sample was Cryopreserved Mouse Cortical Neural Stem Cells (Cat No.SCR029, Sigma-Aldrich, St. Louis, MO, USA), cultured using Mouse Stem Cell Expansion Media (Cat No.SCM008, MilliporeSigma, Burlington, MA, USA). The treatment groups are using Curcumin (Cat No.C7727, Sigma-Aldrich) with several concentrations ranging from 0.1 μM, 0.5 μM, one μM, and two μM. Synthetic growth factors (beta Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), and heparin) stimulated stem cell proliferation as the positive control group, and the negative control group using Dimethyl Sulfoxide (DMSO) solution. The research permitted by Universitas Esa Unggul Research Ethics Commission (No. 0161-21.161/dpke-kep/final-ea/ueu/vi/2021).

2.2 Method

2.2.1 Stem Cell Culture

Cultured two days of Neural Stem cells in T75 flasks coated by 10 mg/mL poly-l-ornithine (Cat No.P3655-10MG, Sigma-Aldrich) and laminin (Cat No.CC095, Sigma-Aldrich) and incubated overnight in a 5 percent CO2 incubator at 37°C. The coated flask by Poly-l-ornithine was re-coated with laminin with a final concentration of 7 g/mL, dissolved using DPBS the next day.

We used Neural Stem Cell Basal Medium (Cat No.SCM014, Sigma-Aldrich) for cell culture of mouse-derived neural stem cells without growth factors and serum medium as a negative control, medium enriched with growth factor bFGF (Cat No.T2815-2UG, Sigma-Aldrich) using for the positive control, EGF (Cat No.SAB4200802 Sigma-Aldrich) 25μL, and Heparin (Cat No.H3149-10KU, Sigma-Aldrich) each concentration is 100 μg/mL.

2.2.2 Passaging Cells

Passage cells using 3 mL of Accutase solution (Cat. No. SCR005, Sigma-Aldrich) and incubating in an incubator at 37°C for 3 Min, and moved to T75 Flask. The dissociated cells were transferred into a conical tube and centrifuged at 300 x g for 2-3 Min. We discarded the supernatant to obtain pellets into the conical and added 2 mL of Neural Stem Cell Basal Medium containing growth factor. The cells were stained by trypan blue (Cat No.CC095, MilliporeSigma) and counted by a hemocytometer.

The research method for herbal Curcumin dissolved in DMSO at 0.1percent (0.1 μM; 0.5 μM; 1 μM; and two μM) testing was by administering basal medium and enrichment medium with growth factors FGF, EGF, and heparin in cell culture after side thawing of passage 2. The positive control used synthetic growth, and the negative control group used a DMSO solution without growth factor in the basal medium of NSC at passage 3-D0 (P3D0), where the cell differentiation begins.

2.2.3 WST-1 Assay

We used the WST-1 assay to analyze the proliferation cell by Cell Proliferation Reagent WST-1 kit (Cat No.05015944001, Sigma-Aldrich) at passage 3-D4 (P3D4). Cell cultures were added to the microplate at a concentration of 4x104 cells/well in 100 μL of culture medium at passage 3- D3 (P3D3). A reagent of WST-1 as 10μL/well was added and incubated for 4H at 37°C and 5 percent CO2.

2.2.4 Gene Expression

We isolated RNA from the cells using RNA extraction from the GenElute. Total RNA purification kit (Cat No.RNB100, Merck, Kenilworth, NJ, USA) and amplified RNA samples using KAPA Probe Fast One-step (Cat No.KK4752, Merck).

2.2.5 Statistical Analysis

We used the SPSS ver. 26 software program (IBM Corporation, Armonk, NY, USA) for the normality test for all data, one-way ANOVA comparison tests, and the Tukey HSD post hoc test.

3. RESULTS AND DISCUSSION

The viability rate of cells is based on the total number of cells minus the number of dead cells and divided by the total number of cells (Figure 1). The cell culture results up to passage 3 showed an increase in the number of cells to obtain a percentage (Figure 2). Cell viability reached 97.48 percent at passage 3. Cell cultures with viability > 80 percent can treat proliferation and differentiation tests.

The morphology of cultured NSC cells using NSC Complete Medium was enriched with growth factors (FGF, EGF, and Heparin) from 0 to 2 passages before adding the curcumin treatment (Figure 3). On day 3, we observe the cells when the cell begins to differentiate into fibroblast-like cells at every passage. Cell differentiation began into fibroblast-like cells, condensing from passage 0 to 2 on D3.

Morphological cell analysis revealed that neurosphere and fibroblast-like cells appeared in the positive group at P3D3. Cells grown in a growth factor-enriched medium, EGF or FGF-2, or both demonstrated high levels of cell proliferation activation. Heparin stimulates the binding of bFGF with its receptor on cells and increases the stability of EGF. [16][17]

In the 0.5 μM curcumin treatment group, neuron cells proliferate to form neurospheres. The density cell in the curcumin treatment group is denser than the positive control. In this observation, the neurosphere appears at the P3D3. The neurosphere is the precursor for neuron cells that differentiate into adult neuron cells (astrocyte, neuron, and neuroglia). Curcumin concentrations are more significant than five μM inhibited NSC proliferation. Bang (2018) investigated the proliferative ability of NSPC stimulated by curcumin at a concentration of less than one μM. Curcumin affects SC-NSPC proliferation as evidenced by an increase in NSPC proliferation and formation of astrogliosis originating from the neurosphere after 72 hours of curcumin treatment at curcumin doses of 0.1 and 1 μM.[7][18]

3.1 Curcumin extract treatment

Figure 4 shows the morphology of cultured cells in the Curcumin group, DMSO group, and synthetic growth factor group. After the culture reaches 97.48 percent confluence at passage 3, add the culture by curcumin extract, DMSO, and artificial growth factor. The morphology cell was undifferentiated in the DMSO group and the two μM treatments. At the same time, the treatment group of curcumin 0.5 μM and the synthetic growth factor group showed denser cell growth and formed a neurosphere and fibroblast-like cells. In the group of 0.1 μM and one μM curcumin treatment, cell growth was more tenuous but had formed neurosphere and fibroblast-like cells (Figure 4).

3.2 WST-1 Assay

WST-1 test analysis performed on the 3rd passage of proliferating mouse NSC cells resulting from the herbal treatment of Curcumin at a concentration of 0.1 μM, 0.5 μM, one μM, and two μM treatments, DMSO, and synthetic growth factor treatment. Proliferating cells will absorb fluorescent light and appear colored with different OD values, indicating the number of proliferating cells and analyzed by calculating the stimulation index of cell proliferation (Figure 5).

The Shapiro-Wilk statistical test results showed that increasing cell presentation was distributed normally ($p > 0.05$). The one-way ANOVA and Tukey post hoc led to a significant difference between the positive control (synthetic growth factor) group and the other treatment groups (except 0.5 μM).

The bar chart in Figure 5 is the index stimulation of proliferation cells. The cell proliferation index in the 0.5 μM treatment group also differed from the other curcumin treatment groups and the negative control group. The 0.5 μM treatment and positive control groups were not significantly different.

The results of statistical tests on the distribution of normality data on the number of proliferating cells showed a p-value > 0.05, which indicated data distributed normally. The one-way ANOVA and Tukey post hoc test results showed that the WST-1 assay cell proliferation by index stimulation was not significantly different between the Curcumin treatment group (0.1 μM, one μM, and two μM) and the DMSO group. Significant differences occurred between the positive control and all treatment groups, except with the 0.5 μM treatment group. The positive control group and the 0.5 μM group were not significantly different. The result of the

proliferation cell shows that the positive control group and the 0.5 uM curcumin treatment group have the same ability.

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3.3 The gene of marker NSC expression

The *SOX2, MAP-2*, and *Nestin* gene expression measured by the qPCR assay showed that the SOX2 gene expression was relatively higher than the control (Figure 6). Meanwhile, the expression of *MAP-2* and *Nestin* genes was lower than the negative control relatively. The comparison of *Nestin, MAP-2, SOX2*, and *18S* gene expression in the curcumin treatment group and the positive control in Figure. 6. Each curcumin treatment group's *MAP-2* and *Nestin* genes showed lower expression than the *18S* gene. *Sox2* gene expression in each curcumin treatment group, including the positive control group, appears somewhat higher than the expression of the *18S* control gene, *MAP-2* gene, and *Nestin* gene.

The data of gene expression was distributed normally by the Shapiro-Wilk test (p > 0,05). The one-way ANOVA and Tuckey posthoc tests showed that the expression gene control (18S gene) and the *Sox2, MAP-2*, and *Nestin* expression genes in each group treatment were different. *Sox2* gene expression difference with M

Marker gene expression (*MAP-2, Nestin*, and *SOX2*) in the Curcumin treatment group showed that all the gene expressions reached a higher peak in the 0.5 µM concentration by qPCR assay (Figure 6). The results indicate that the concentration of 0.5 µM curcumin is the appropriate concentration to stimulate the proliferation of mouse cell NSCs so that the markers of cell proliferation genes reach their peak at this concentration. *SOX2* gene expression showed relatively higher than the control 18S gene as a housekeeping gene. *MAP-2* and *Nestin* genes' mRNA expression was lower than the 18S gene. The one-way ANOVA and Tukey's post hoc tests showed a significant difference between the expression of the *SOX2* gene and the expression of other genes. *SOX2* protein has a substantial role in the developmental center (CNS) and peripheral nervous system (PNS) by controlling the proliferation and differentiation of fetal progenitor cells. *SOX2* expression is also critical for retina proliferation and differentiation of neural progenitor cells. The *SOX2* gene is the marker of regulating stem cell self-renewal and maintaining pluripotency.[11]

The low expression of the *MAP-2* gene in our results indicates that the neuronal cell proliferation at passage 3, day three, has not yet reached cell maturation. At the maturation stage, the *MAP-2* gene increases, which indicates that the cell has entered the maturation stage.[13] Tian studied the expression of *Nestin* and *MAP-2* using Western blot one week after the Sprague-Dawley rats' spinal cord injury. *Nestin* is found distal to the axon and not at the dendritic ends. *Nestin* regulates the dynamics of neuron cell growth.[19] Our study showing the low expression of *Nestin* proved that the neurons we studied had not yet matured into adult neurons with axons. Still, neurosphere morphology was formed at a dose of 0.5 µM curcumin, indicating the development process of adult neurons. The neurosphere begins the formation of adult neurons.[20]

Curcumin can act as a growth factor that can stimulate the proliferation of cortical neuronal sel (NSCs). The results show that curcumin is a growth factor because of its success in stimulating the growth of NSC cells. The results showed that the herbal extract curcumin with a concentration of 0.5 µM was the best for promoting the NCSs' proliferation and differentiation. Adult rat neural stem/progenitor cells survived and could increase at 0.5 µM curcumin concentration after 72 hours of treatment.[7] Gersey (2017) studied the significant increase of proliferative Neural Stem cells and newly formed neurons in the mouse hippocampus with a decreased number of apoptotic neurons after being given additional curcumin treatment via the Notch signaling pathway. [15]

4. CONCLUSIONS

The results indicate that curcumin is a growth factor in the growth of Cortical Neuron Stem Cell cells as synthetic growth factors. The optimal concentration of curcumin capable of stimulating cell proliferation is 0.5 µM. The WST-1 test showed the highest number of proliferative cells at a concentration of 0.5 µM, and the gene markers of proliferating neuron cells (SOX2, MAP-2, and Nestin) peaked at this concentration.

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REFERENCES

[1] G. Castelo-Branco *et al.*, "Neural stem cell differentiation is dictated by distinct actions of nuclear receptor corepressors and histone deacetylases," *Stem Cell Reports*, vol. 3, no. 3, pp. 502–515, 2014, doi: 10.1016/j.stemcr.2014.07.008.

[2] A. L. Pang *et al.*, "Neural stem cell transplantation is associated with inhibition of apoptosis, Bcl-xL upregulation, and recovery of neurological function in a rat model of traumatic brain injury," *Cell Transplant.*, vol. 26, no. 7, pp. 1262–1275, 2017, doi: 10.1177/0963689717715168.

[3] G. Tejeda, A. J. Ciciriello, and C. M. Dumont, "Biomaterial Strategies to Bolster Neural Stem Cell-Mediated Repair of the Central Nervous System," *Cells Tissues Organs*, 2021, doi: 10.1159/000515351.

[4] L. Li *et al.*, "SoxD genes are required for adult neural stem cell activation," *Cell Rep.*, vol. 38, no. 5, 2022, doi: 10.1016/j.celrep.2022.110313.

[5] A. Chouw *et al.*, "Ischemic Stroke: New Neuron Recovery Approach with Mesenchymal and Neural Stem Cells," *Mol. Cell. Biomed. Sci.*, vol. 2, no. 2, p. 48, 2018, doi: 10.21705/mcbs.v2i2.28.

[6] I. Arundina, K. Suardita, I. Diyatri, and M. Dwi, "Journal of International Dental and Medical Research ISSN 1309-100X Ira Arundina and et al Volume • 11 • Number • 3 • 2018 Experimental article," 2018. [Online]. Available: http://www.jidmr.com.

[7] W. S. Bang, K. T. Kim, Y. J. Seo, D. C. Cho, J. K. Sung, and C. H. Kim, "Curcumin increase the expression of neural stem/progenitor cells and improves functional recovery after spinal cord injury," *J. Korean Neurosurg. Soc.*, vol. 61, no. 1, pp. 10–18, 2018, doi: 10.3340/jkns.2017.0203.003.

[8] Peng Zhao, X. Su, T. Ge, and J. Fan, "乳鼠心肌提取 HHS Public Access," *Physiol. Behav.*, vol. 176, no. 1, pp. 139–148, 2016, doi: 10.1016/j.antiviral.2019.04.011.Curcumin.

[9] G. M. Liu, K. Xu, J. Li, and Y. G. Luo, "Curcumin upregulates S100 expression and improves regeneration of the sciatic nerve following its complete amputation in mice," *Neural Regen. Res.*, vol. 11, no. 8, pp. 1304–1311, 2016, doi: 10.4103/1673-5374.189196.

[10] J. T. Velasquez *et al.*, "Low-dose curcumin stimulates proliferation, migration and phagocytic activity of olfactory ensheathing cells," *PLoS One*, vol. 9, no. 10, 2014, doi: 10.1371/journal.pone.0111787.

[11] K. Arnold *et al.*, "Sox2 + adult stem and progenitor cells are important for tissue regeneration and survival of mice," *Cell stem cells*, vol. 9, no. 4Cell Stem Cell, pp. 317–329, 2011, doi: 10.1016/j.stem.2011.09.001.

[12] S. Suzuki, J. Namiki, S. Shibata, Y. Mastuzaki, and H. Okano, "The neural stem/progenitor cell marker nestin is expressed in proliferative endothelial cells, but not in mature vasculature," *J. Histochem. Cytochem.*, vol. 58, no. 8, pp. 721–730, 2010, doi: 10.1369/jhc.2010.955609.

[13] M. H. Mohammad, A. M. Al-Shammari, A. A. Al-Juboory, and N. Y. Yaseen, "Characterization of neural stemness status through the neurogenesis process for bone marrow mesenchymal stem cells," *Stem Cells Cloning Adv. Appl.*, vol. 9, pp. 1–15, 2016, doi: 10.2147/SCCAA.S94545.

[14] F. Attari *et al.*, "Curcumin as a double-edged sword for stem cells: Dose, time and cell type-specific responses to curcumin," *DARU, J. Pharm. Sci.*, vol. 23, no. 1, pp. 2703–2706, 2015, doi: 10.1186/s40199-015-0115-8.

[15] Z. C. Gersey *et al.*, "Curcumin decreases malignant characteristics of glioblastoma stem cells via induction of reactive oxygen species," *BMC Cancer*, vol. 17, no. 1, pp. 1–11, 2017, doi: 10.1186/s12885-017-3058-2.

[16] X. Li *et al.*, "EGF and curcumin co-encapsulated nanoparticle/hydrogel system as potent skin regeneration agent," *Int. J. Nanomedicine*, vol. 11, pp. 3993–4009, 2016, doi: 10.2147/IJN.S104350.

[17] K. Mizukoshi, N. Koyama, T. Hayashi, L. Zheng, S. Matsuura, and M. Kashimata, "Shh/Ptch and EGF/ErbB cooperatively regulate branching morphogenesis of fetal mouse submandibular glands," *Dev. Biol.*, vol. 412, no. 2, pp. 278–287, 2016, doi: 10.1016/j.ydbio.2016.02.018.

[18] D. K. Kim, J. In Kim, B. R. Sim, and G. Khang, "Bioengineered porous composite curcumin/silk scaffolds for cartilage regeneration," *Mater. Sci. Eng. C*, vol. 78, pp. 571–578, 2017, doi: 10.1016/j.msec.2017.02.067.

[19] T. Tian, Y. Zhang, S. Wang, J. Zhou, and S. Xu, "Sox2 enhances the tumorigenicity and chemoresistance of cancer stem-like cells derived from gastric cancer," *J. Biomed. Res.*, vol. 26, no. 5, pp. 336–345, 2012, doi:

10.7555/JBR.26.20120045.
[20] L. Zhang *et al.*, "The necessity for standardization of glioma stem cell culture: A systematic review," *Stem Cell Res. Ther.*, vol. 11, no. 1, pp. 1–7, 2020, doi: 10.1186/s13287-020-01589-8.