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Curcumin Induces the Cell Proliferation 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). We studied the ability of curcumin to stimulate neural stem cell proliferation to obtain optimal doses of cell proliferation. The concentrations of curcumin in this study were 0.1 µM, 0.5 µM, one µM, and two µM. The positive controls used were Synthetic Growth Factor Basic Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), and heparin. The dimethyl Sulfoxide (DMSO) solution was used as a control negative. Analysis of NSC proliferation ability using the WST-1 (Water Soluble tetrazolium) test. RT-PCR test is used to analyze the expression of neural cell proliferation marker genes (MAP-2 gene, Nestin gene, and SOX2. Morphological results and gene expression analysis showed that the cells proliferated optimally at 0.5 µM curcumin dose. One-way ANOVA analysis test and Tukey posthoc test results ($p < 0,05$) in the WST-1 test showed that 0.5 µM curcumin concentration was the optimal concentration to stimulate cell proliferation. The results of gene expression of Sox2, MAP-2, and Nestin showed thar the highest espression was occurred at 0.5 µM curcumin. The results of this study show that a curcumin concentration of 0.5 µM can stimulate NSC cell proliferation like stimulation by synthetic growth factors.

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

Abstrak
Penelitian mengenai herbal kurkumin masih terus berkembang, salah satunya perannya dalam merangsang proliferasi dan diferensiasi neural stem cell (NSC). Pada penelitian ini digunakan kurkumin untuk merangsang proliferasi sel induk saraf untuk memperoleh dosis optimal proliferasi sel. Konsentrasi kurkumin yang kami gunakan pada penelitian ini adalah 0,1 µM, 0,5 µM, 1 µM, dan 2 µM. Kontrol positif yang digunakan adalah Synthetic Growth Factor Basic Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), dan heparin. Sebagai kontrol negatif digunakan larutan Dimetil Sulfoksida (DMSO). Analisis kemampuan proliferasi NSC menggunakan uji WST-1 (Water Soluble tetrazolium dan uji RT-PCR dengan menganalisis ekspresi gen penanda proliferasi sel saraf (gen MAP-2, gen Nestin, dan SOX2. Hasil Analisis morfologi dan ekspresi gen menunjukkan bahwa sel berproliferasi optimal pada dosis kurkumin 0,5 µM. Analisis one way ANOVA dan hasil uji posthoc Tukey ($p < 0,05$) pada uji WST-1 menunjukkan bahwa konsentrasi kurkumin 0,5 µM merupakan konsentrat yang optimal untuk merangsang proliferasi sel. Hasil uji ekspresi gen Sox2, MAP-2, dan Nestin tertinggi terjadi pada kurkumin 0,5 µM. Maka hasil penelitian tesebut menunjukkan bahwa konsentrasi kurkumin 0,5 µM dapat merangsang proliferasi sel NSC seperti stimulasi oleh faktor pertumbuhan sintetik.

Kata Kunci: Ekspresi gen, gen MAP, gen Nestin; qPCR; gen SOX-2

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. Some studies have shown that neural stem cells have limitations in neurogenesis [4][2]. 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][7]. Curcumin is an alternative natural growth factor because it stimulates cell proliferation and differentiation [8][9]. 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 [10]. Pre-research results show that the dose of 0.2 mg/kg curcumin can activate NSC proliferation, increase neurogenesis, and improve cognitive impairment in rat animal models [11].

During the proliferation and differentiation process, embryonic stem cells and adult NSCs will express high levels of the SOX2 gene [12]. 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 [8][13]. The MAP-2 gene plays a role in microtubule assembly and the process of neurogenesis [14].

Curcumin has the active ingredient diferuloyl-methane, which has many biological effects, including anti-inflammatory, antioxidant effects, and cell apoptosis [15][10]. 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 [16].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 q-PCR method.

METHODS

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, Millipore Sigma, 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).

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.

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 DMSO solution without growth factor in the basal medium of NSC at passage 3-D0 (P3D0), where the cell differentiation begins.

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.

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).

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.

RESULT

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.

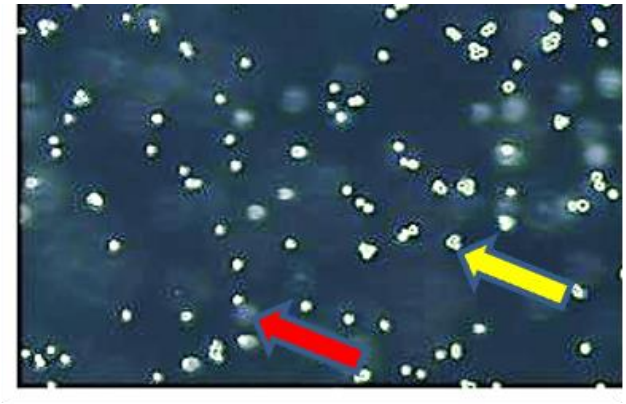

Figure 1 In the WST-1 assay, the tetrazolium salt WST-1 was cleaved to formazan by glycolytic NAD(P)H in living cells and scanned by a spectrophotometer. Live cells appear to glow with a spectrophotometer scan (yellow arrow). The dead cells (red arrow) do not fluoresce formazan dye due to NAD(P)H glycolysis. WST-1 assay performed at 3rd passage. Magnification 100X; White bar: 100 µm.

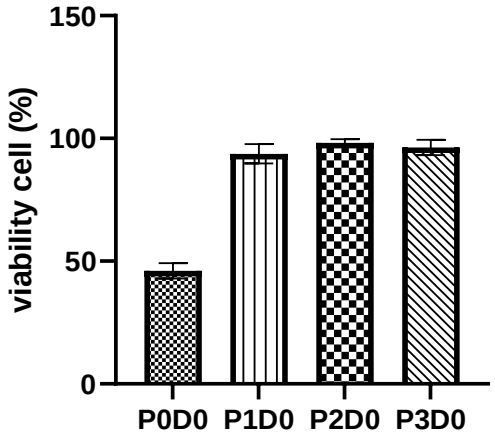

Figure 2. Percentage of NSC cell viability (%) from 0 passage to 3 passage on day 0. P: passage; D: day.

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 [17][18].

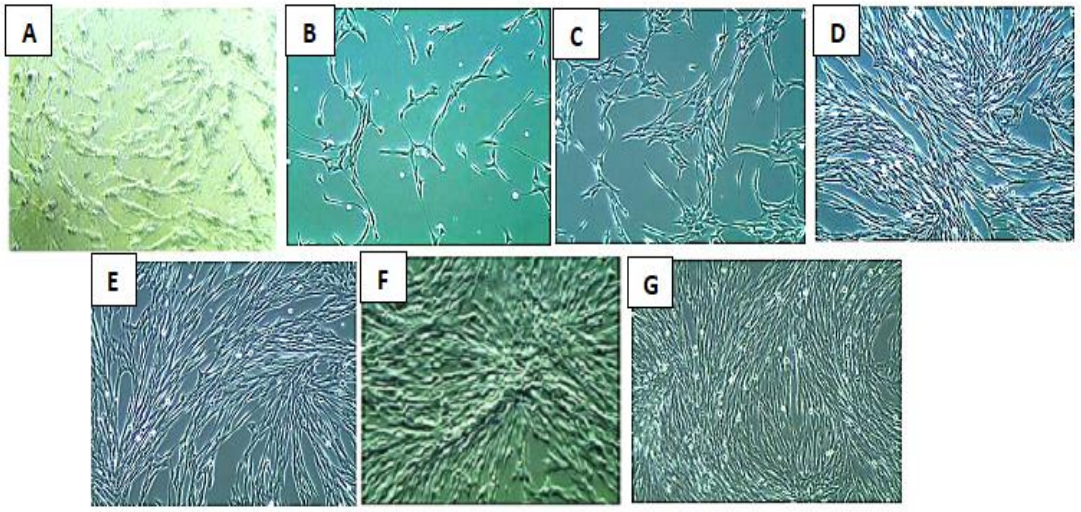

Figure 3. The morphology of the NSC cells cultured after the thawing process in several parts before passage, with the number of seeding cells, was about 2 million. (A). on passage 0, day 3 (P0D3), fibroblast-like cell (red arrow); (B). on passage 1, day 3 (P1D3), fibroblast-like cell (red arrow); (C). on passage 1, day 4 (P1D4), fibroblast-like cell (red arrow); (D). on passage 1, day 5 (P1D5); (E). on passage 2, day 1 (P2D1); (F). on passage 2, day 2 (P2D2); (G). on passage 2, day 3 (P2D3). Black bar: 100 µm.

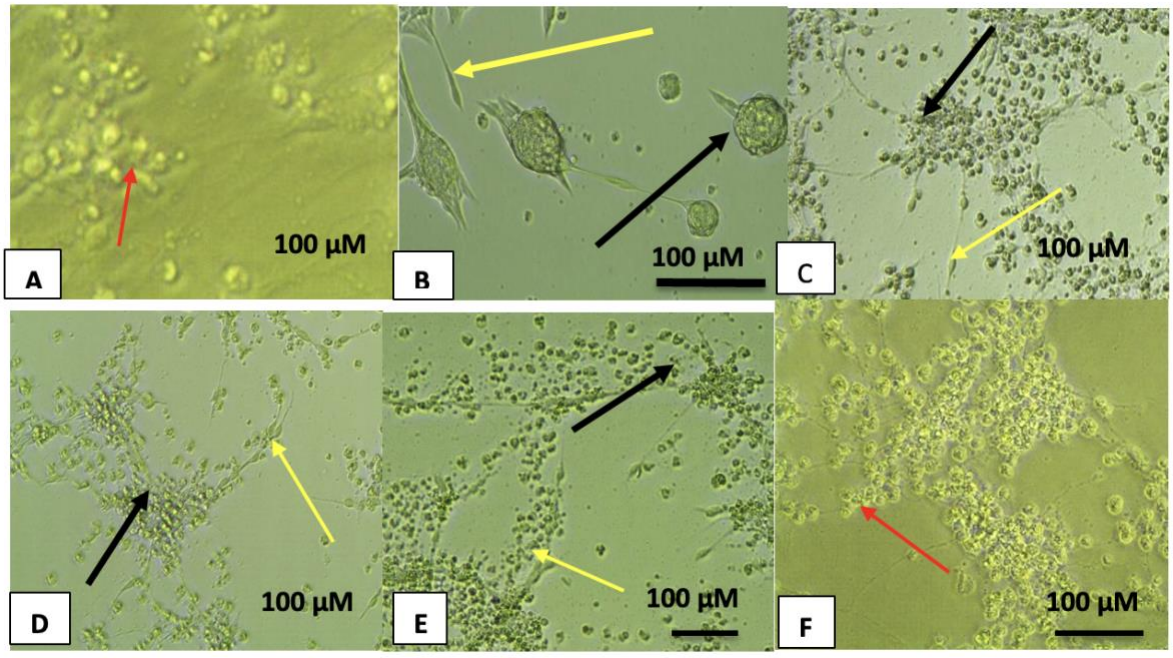

Figure 4. The group's morphology of NSC cells differed on day three after curcumin treatment. (A) Undifferentiated cells (red arrow), Negative control; (B) neurosphere (black arrow) and fibroblast-like cells (yellow arrow), Positive control; (C) neurosphere (black arrow) and fibroblast-like cell (yellow arrow), 0,1 µM Curcumin group ; (D) neurosphere (black arrow) and fibroblast-like cells (yellow arrow), 0.5 µM Curcumin group ; (E) neurosphere (black arrow) and fibroblast-like cells (yellow arrow), 1 µM Curcumin group; (F) undifferentiated cell (red arrow), 2 µM Curcumin group. Black bar: 100 µm. (Magnification A,C-F: 100X and B 400X)

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).

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.

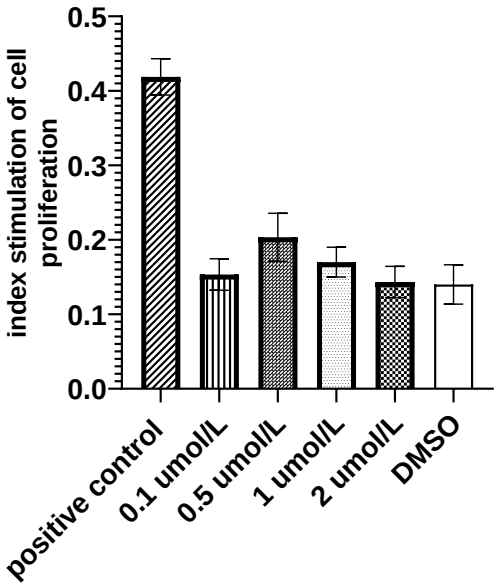

Figure 5. Bar graph of stimulation Index of NSC Cell Proliferation by WST-1 assay test in mouse NSC cell culture.

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.

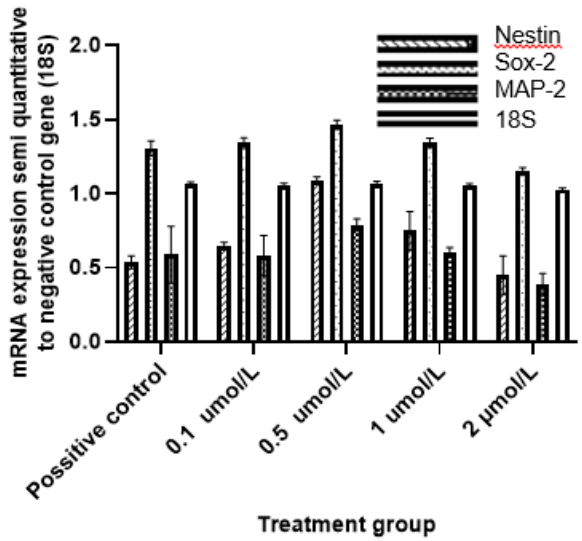

Figure 6. Comparison of *MAP-2* gene mRNA expression. *Nestin, Sox2*, and *18S* in each curcumin treatment group and positive control group

DISCUSSION

In the 0.5 µM curcumin treatment group, neuron cells proliferated 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 treatment at 0.1 and 1 µM doses [8][19].

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 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 [12][1].

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.[14]

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 [20][21]. 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 [22].

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 [8][23]. 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 [16].

CONCLUSION

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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ETICAL STATEMENT

This study was approved by ethical committee Universitas Esa Unggul Research Ethics Commission (No. 0161-21.161/dpke-kep/final-ea/ueu/vi/2021).

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