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Curcumin Regulation of Oligodendrocyte Differentiation and Development

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CURCUMIN REGULATION OF OLIGODENDROCYTE DIFFERENTIATION AND DEVELOPMENT

BY

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Abstract

Curcumin is a naturally obtained hydrophobic polyphenol that is under investigation for its potential benefits in multiple sclerosis (MS) treatment. MS development involves two main stages in its pathophysiology – the establishment and progression of inflammation that causes blood and brain barrier (BBB) damage, followed by neurodegenerative mechanisms that result in myelin sheath disruption and oligodendrocyte apoptosis. These immunopathological and neurodegenerative processes lead to disrupted remyelination in MS, which has been previously associated with the oligodendrocyte progenitor cells (OPCs). Specifically, disrupted OPC differentiation has been hypothesized to have a large role in impaired remyelination in MS. Curcumin's immunoregulatory, anti-apoptotic, anti-oxidant, and anti-degenerative activity make it an effective candidate for targeting both the autoimmune and the neurodegenerative stages of MS. Primarily, our research focused on identifying curcumin's role in oligodendrocyte differentiation and development *in vitro* through two study populations – mixed glial cultures and OPC cultures. Our findings showed a significant decrease in the total cell number and a trending decrease in differentiation in mixed glial cultures. Additionally, we found a significant increase in oligodendrocyte differentiation in response to 1 μM curcumin treatment in OPC cultures. Our overall results suggest that curcumin may have potential differentiation-enhancing activity in oligodendrocytes. Further studies are needed to investigate curcumin's differentiation-enhancing effect in heterogenous and homogenous cultures *in vitro* and *in vivo,* as well as to identify the signaling pathways involved in curcumin's regulation of differentiation in oligodendrocytes.

Introduction

Curcumin is a hydrophobic polyphenol that can be naturally obtained from *Curcuma longa* plant roots as one of the active components of turmeric (Ghanaatian et al. 2018). This natural compound has been largely studied for its potential therapeutic regulation of neurodegenerative disorders, including multiple sclerosis (MS) (Qureshi et al. 2018). MS is a demyelinating and neurodegenerative disorder of the Central Nervous System (CNS) that affects around 2.8 million individuals worldwide (Haider et al. 2016; Walton et al. 2020). The continuously rising global prevalence of MS since 2013 presents the importance of understanding MS etiology and developing new treatments or a cure that targets its pathophysiology (Walton et al. 2020).

While the causes and origins of MS are still unknown, several primary research studies have shown the role of autoimmunity in disease progression (Ghanaatian et al. 2018). The neuroinflammatory processes associated with MS development show the involvement of both the innate and the adaptive immune system cells (Haider et al. 2016). Studies done in experimental autoimmune encephalomyelitis (EAE) animal models of MS revealed the crucial role of various immune system cells (Th17, Th1, Th9), inflammatory cytokines and interleukins (IL-7, IL-17, IL-22, IL-23), and proinflammatory cytokines (TNF-α) in the development of MS (Qureshi et al. 2018). Cytokines are a broad group of proteins produced by T cells and macrophages that have an active role in establishing communication between cells and often lead to a series of cascade reactions (Zhang & An, 2007). This group includes chemokines, interleukins, proinflammatory and inflammatory cytokines that have the same functional role but belong to distinct categories based on the differences in their mechanisms of activity (Zhang & An, 2007). Specifically, proinflammatory cytokine activity induces

inflammatory reactions by activating other inflammatory cytokines and establishing inflammation in the system (Kany et al. 2019).

The immunopathologic processes in MS result in lesion formation that damages the blood and brain barrier (BBB) (Jadidi-Niaragh & Mirshafiey, 2011). The BBB disruption is primarily associated with the inflammatory processes caused by T cells, which activate several proinflammatory and inflammatory cytokines (Ghanaatian et al. 2018). These immunopathologic changes cause the inflammatory cascade that establishes the first stage of MS pathology (Ghanaatian et al. 2018). The immunopathology in MS induces axonal impairment and loss, and causes myelin sheath degradation, and oligodendrocyte damage and apoptosis in the CNS (Jadidi-Niaragh & Mirshafiey, 2011). These degenerative events establish the second stage of MS resulting in its clinical symptoms (Ghanaatian et al. 2018). Thus, previously developed therapeutic approaches for MS utilize involve both anti-inflammatory and anti-degenerative compounds that can target either or both stages of MS pathology (Ghanaatian et al. 2018).

Previous literature has identified curcumin's functional activity in immunoregulation, anti-degenerative processes, and neuroprotective mechanisms as a natural compound (Qureshi et al. 2018). Curcumin has especially received wide attention due to its two significant properties for targeting neurological and neurodegenerative disorders – curcumin's extensive biochemical effects in the body and its common use outside of the clinical setting *via* incorporating turmeric in cooking (Qureshi et al. 2018). Curcumin's activity has been previously studied in large-scale analyses of neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), and Multiple sclerosis (MS), as well as Huntington's disease as a neurological disorder (Qureshi et al. 2018). These studies

show the wide range of curcumin's biochemical activity which involves multiple intrinsic molecular targets (Qureshi et al. 2018). For instance, curcumin has shown inhibitory activity in several transcription factors and enzymes such as nuclear factor-κB (NF-κB) which has an important role in immunity and inflammation regulation (Zhou et al. 2011). Curcumin has also shown the ability to downregulate the production of the proinflammatory cytokine TNF-α, which is extensively involved in the progression of systemic inflammation (Zhou et al. 2011). The regulation of NF-κB and other transcription factors by curcumin, as well as its ability to downregulate proinflammatory cytokines, supports its hypothesized inhibitory activity towards neuroinflammatory processes (Qureshi et al. 2018). Additionally, curcumin has shown anti-oxidant activity in PD models through oxidative stress reduction *via* α-synuclein and monoamine oxidase B inhibition (Qureshi et al 2018). Previous *in vivo* studies have further suggested curcumin's neuroprotective activity and its ability to induce neuroplasticity through its regulation of antioxidant enzymes and heat shock proteins (Qureshi et al. 2018). These findings support curcumin's extensive activity due to its involvement in multiple biochemical processes and pathways in neurodegenerative and neurological disorders.

Similar findings have been previously identified in both *in vivo* and *in vitro* studies focused on curcumin's role in MS pathophysiology. Curcumin has been shown to reduce inflammatory cells and proinflammatory cytokines (IL-6, IL-1β, TGF-β) in *in vivo* studies conducted in EAE Lewis rats (Qureshi et al. 2018). The suppression of proinflammatory cytokines has been shown to be regulated by NF-κB mediation *in vitro* in dendritic cells, which supports the findings mentioned for other neurodegenerative disorders (Qureshi et al. 2018). Curcumin has also shown anti-apoptotic activity that targets the mitochondrial

dysfunction caused by MS pathophysiology (Qureshi et al. 2018). This anti-apoptotic activity has been demonstrated through *in vivo* studies that showed alleviation of EAE following curcumin treatment *via* several mechanisms, such as inhibition of the JAK-STAT pathway, and regulation of T cells and toll-like receptors (TLRs) (Qureshi et al. 2018). Moreover, curcumin treatment has shown the ability to attenuate oligodendrocyte apoptosis *in vivo* due to antioxidant mechanisms that reduce mitochondrial and endoplasmic reticulum stress by targeting intrinsic apoptotic pathways (Qureshi et al. 2018). Thus, curcumin's extensive biochemical activity is a strong candidate for further consideration for MS therapy through both *in vivo* and *in vitro* studies. Moreover, in addition to its extensive activity through multiple targets, curcumin is a natural, plant-based compound that is known to have less toxicity, fewer severe side effects, and higher effectiveness than currently available therapeutic treatments for MS (Ghanaatian et al. 2018). Thus, there is extensive interest in incorporating a natural compound such as curcumin into newly developing MS treatments (Qureshi et al. 2018).

Remyelination is the repair mechanism in the CNS in response to demyelinated axons observed in demyelinating diseases, including MS (Chari, 2007). Despite some remyelination observed in MS lesions, it is still highly limited in most of the actively demyelinating regions (Kuhlmann et al. 2008). While causes for failed remyelination in MS are unknown, previous research has indicated the possible role of oligodendrocyte progenitor cells (OPCs) in these processes (Kuhlmann et al. 2008). Specifically, the absence of necessary OPCs in demyelinating regions, their failure to differentiate, and/or their inability to communicate with axons may cause failure in remyelination (Kuhlmann et al. 2008). Thus, it is significant to consider the complex mechanisms involved in the differentiation of OPCs to myelinating

oligodendrocytes to understand and target the pathophysiology of MS (Guardiola-Diaz et al. 2012).

Oligodendrocytes have distinct morphological and biochemical characteristics that distinguish their progression through their stages of maturation both in *in vivo* and *in vitro* studies (Baumann & Pham-Dinh, 2001). These maturation stages are known to advance in a specific order as shown in Figure 1 – the differentiation of bipolar early progenitor cells to multipolar late progenitors is followed by their terminal differentiation into immature oligodendrocyte cells that branch out and form into mature oligodendrocytes (Guardiola-Diaz et al. 2012). Guardiola-Diaz et al. (2012) showed the activity of two distinct pathways – Ras/Raf/Mek/Erk and PI3K/Akt/mTOR pathways – in oligodendrocyte differentiation in a sequential but non-overlapping manner. Erk1/2 signaling displayed a regulatory effect on the transition from early to late progenitors followed by differentiation to immature oligodendrocytes (Guardiola-Diaz et al. 2012). In turn, mTOR signaling regulation showed an effect on the transition of immature oligodendrocytes to mature oligodendrocytes (Guardiola-Diaz et al. 2012). Thus, it is necessary to consider these signaling processes for the development of therapeutic approaches that promote remyelination in MS by inducing OPC differentiation (Guardiola Diaz et al. 2012).

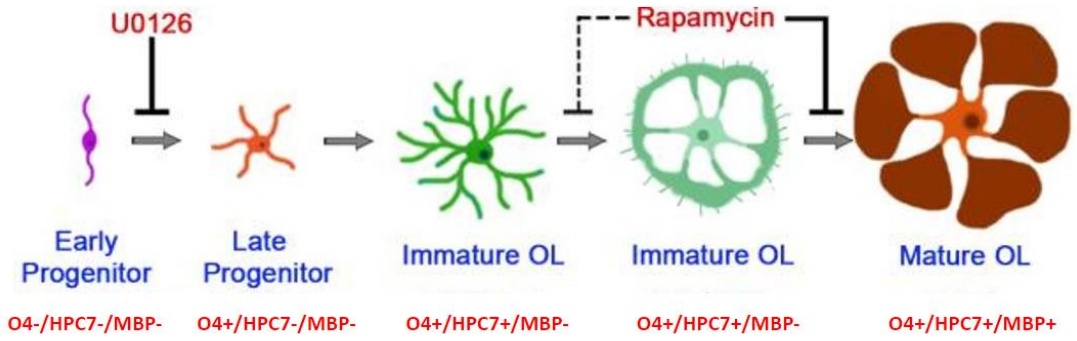

Figure 1: The oligodendrocyte cell lineage represented through their morphological and biochemical characteristics. The distinct stages in the oligodendrocyte cell lineage are identified based on the expression of specific antigenic markers. O4 is a cell surface marker that is expressed starting from the late progenitor stage of the cell lineage and is indicative of the loss of oligodendrocyte bipolar morphology (Kuhn et al. 2019). HPC7 is an oligodendrocyte-specific protein that is expressed as oligodendrocytes transition to the immature oligodendrocyte stage of the cell lineage (Ishii et al. 2012). Myelin basic protein (MBP) is a constituent of myelin and is an indicator of oligodendrocyte transition to the mature oligodendrocyte differentiation stage (Kuhn et al. 2019). The oligodendrocyte cell lineage progression is known to involve the Ras/Raf/Mek/Erk pathway in the early stages of differentiation (shown through the inhibition of Erk1/2 *via* U0126) and the PI3K/Akt/mTOR pathway in the later stages of differentiation (shown through the inhibition of mTOR *via* Rapamycin). Modified from Guardiola-Diaz et al. (2012).

This study examined the role of curcumin on oligodendrocyte lineage progression and differentiation. Additionally, it assessed curcumin's effects that are relevant to the optimal functionality of oligodendrocytes observed through total cell counts, which can account for the potential cytotoxic or anti-proliferative effects of curcumin. In an additional pilot experiment, the study explored an experimental method for directly challenging OPC cultures and identifying the potential protective effects of curcumin in response to a challenge. The study used both mixed glial cultures and purified OPC cultures to analyze the biochemical and morphological effects of curcumin on OPCs through live immunofluorescence microscopy (IFM) and western blotting. A previously identified plate reader methods was used for the pilot study. We hypothesize that curcumin treatment will decrease total cell counts in a concentration-dependent manner due to the anti-proliferative effects in lower curcumin concentrations, and both anti-proliferative and cytotoxic effects in

higher curcumin concentrations. We further hypothesize that curcumin treatment will show differentiation-enhancing effects on both mixed glial cultures and OPC cultures compared to the control. Furthermore, we hypothesize that curcumin treatment will show protective effects in response to a challenge by showing decreased apoptosis in cultures challenged by thapsigargin.

Materials and Methods

Cell cultures

Mixed glial cultures

Neonatal (P1–2) rats were sacrificed according to Trinity College's IACUC approved procedure. Telencephalic hemispheres were isolated and separated, followed by their placement in Hepes-EBSS (HE) buffer. The obtained brain tissue was minced and shaken in a shaking incubator for 20 minutes (110 rpm at 37°C) in trypsin-HE solution. Following the shake, trypsinization was terminated by the addition of a trypsin inhibitor (SBTI) (200 µL /10 mL). After 2 minutes of shaking at room temperature, $MgSO_4$ solution (3mM) and DNase (20 µg/mL) were added and incubated at room temperature for 5 minutes to digest any present extracellular DNA. The resulting solution was evenly split into four 50 mL falcons and centrifuged for 1 minute (2000 rpm). The supernatant was discarded, and the pellet was resuspended in Trituration DNase (80 µg/mL) for 5 minutes. Two new 50 mL conical tubes were obtained – one 50 mL conical tube contained a combined solution of the top 4 mL from each tube and the other 50 mL conical tube combined the rest of 1 mL solution from each tube. Trituration DNase (1 mL) solution was added to the second 50 mL conical tube and left

for 5 minutes to settle. After 5 minutes, the top 4 mL of the second suspension from the 50 mL conical tube were added to the first 50 mL conical tube. The final suspension was centrifuged for 1 minute (2000 rpm). Following the aspiration of the supernatant, the pellet was resuspended in a total of 50 mL 5% fetal bovine serum (FBS) and seeded in poly-L-lysine coated T-75 (2.5×10^7 cells/flask) flasks and poly-L-lysine coated four-well plates (500,000 cells/well). Each T-75 flask contained 15 mL of suspension, and each well contained 0.5 mL of suspension. All T-75 flasks and 4-well plates were incubated at 37°C.

The 4-well plates were used for the live immunofluorescence microscopy (IFM) experiment with mixed glial cultures. Following seeding, cell cultures were fed with 5% FBS every 3-4 days for 11 days. The 5% FBS solution ensured inhibited differentiation in the seeded *in vitro* cultures. On day 11, 5% FBS media was changed with N2 defined media [(DMEM supplemented with human transferrin (50 μg/mL), bovine pancreatic insulin (5 μg/mL), 3,3,5-triiodo-L-thryonine (10 ng/mL), sodium selenium (30 nM), D-biotin (10 ng/mL), hydrocortisone (10 nM), sodium pyruvate (0.11 mg/mL), penicillin-streptomycin (10 IU/mL and 100 μg/mL)] and 1% FBS that allowed for cellular differentiation to take place. This media switch established day 0 (D0) of differentiation. Each well received N2 media with ethanol for the control treatment, or N2 media with 1 μM or 5 μM curcumin for the experimental treatments. The treatment was terminated on day 2 (D2) of differentiation, followed by live IFM and data collection based on staining counts.

Enriched oligodendrocyte progenitor cell (OPC) cultures

Enriched OPC cultures were obtained based on a previously discussed procedure (McCarthy & de Vellis, 1980). As discussed earlier, after obtaining mixed glial cultures and seeding

them on T-75 flasks, the flask cell cultures were fed with 5% FBS solution every 3-4 days for 11 days. After 11 days, an overnight shake was conducted in the orbital shaker (2000 rpm at 37°C) to separate oligodendrocyte progenitor cells (OPCs) from the astrocyte bed. The cultures were further purified through the addition of L-leucine methyl ester solution to release OPCs and remove microglia and other proliferating cells. Enriched and purified OPCs were then seeded on poly-L-Ornithine coated 4-well plates for live IFM, 6-well plates for western blotting, and 96-well plates for the plate reader analysis. The 5% FBS media was switched to serum-free defined N2 medium and 1% FBS to initiate differentiation. The change to the N2 medium initiated the onset of differentiation (D0 of differentiation) and the start of the treatment. Each well in 4-well plates received N2 media with ethanol for the control treatment, or N2 media with 1 µM or 5 µM curcumin for the experimental treatments. The treatment was terminated on day 2 (D2) of differentiation, followed by live IFM staining and data collection. Each well in 6-well plates received N2 media with ethanol for the control treatment, or N2 media with 1 µM curcumin for the experimental treatment. The treatment was terminated on day 2 (D2) of differentiation, followed by western blot analysis. In some experiments for western blot analysis, OPCs were grown in insulin-free N2 medium from D0-D2 of differentiation. These samples were only stimulated with ethanol vehicle or 1 µM curcumin treatment 1 hour prior to sample collection and treatment termination on D2 of differentiation. This experimental treatment was used for analyzing the signaling effects of curcumin in oligodendrocytes in the study.

Experimental design

The experimental design for the study included three main questions for analysis – the effects of curcumin on differentiation (in mixed glial cultures and OPC cultures), on signaling in OPCs, and the protective effects of curcumin in OPCs following a challenge.

Differentiation Analysis

Mixed primary oligodendrocyte cultures comprising oligodendrocytes growing on an astrocyte bed were seeded on 4-well plates without further purification and treated with 1µM or 5 µM curcumin for 48 hours after the establishment of differentiation. The mixed glial cultures were evaluated based on their antigenic markers through a live IFM procedure (Figure 2).

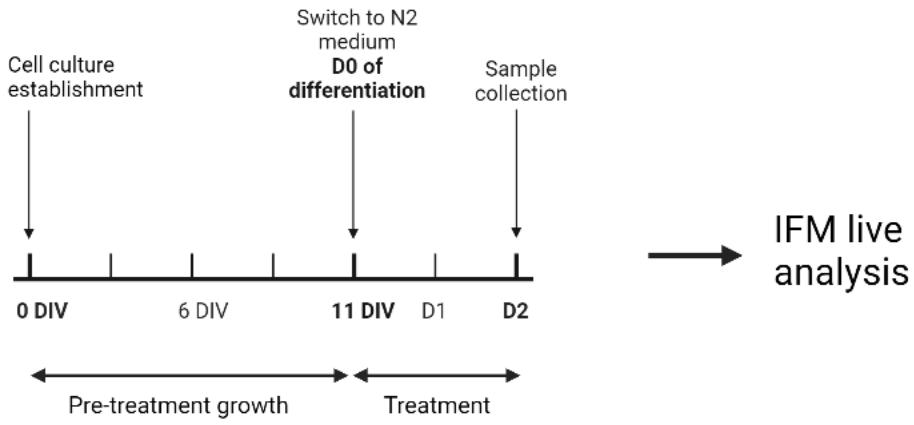

Figure 2: The experimental design for the differentiation analysis in mixed glial cultures *via* live IFM. Created with BioRender.com.

The enriched OPC culture experiments were done on two separately obtained sets of samples – sample set 1 and sample set 2. The two sample sets were analyzed separately to account for the possible differences between the sample sets. The OPCs for both sample sets were seeded

on 4-well plates and 6-well plates, and fed with 5% FBS for 11 days *in vitro* (DIV). On 11 DIV, the 5% FBS medium was switched to a chemically defined serum-free N2 medium containing ethanol as a control vehicle or 1 µM curcumin for the sample set 1 and 1 µM or 5 µM curcumin for the sample set 2. The treatment was continued until Day 2 of differentiation (48-hour treatment). Following treatment termination, the OPC cultures from 4-well plates were evaluated based on their antigenic markers through live IFM staining. The OPC cultures from 6-well plates were collected and stored at –80˚C for further western blot analysis (Figure 3).

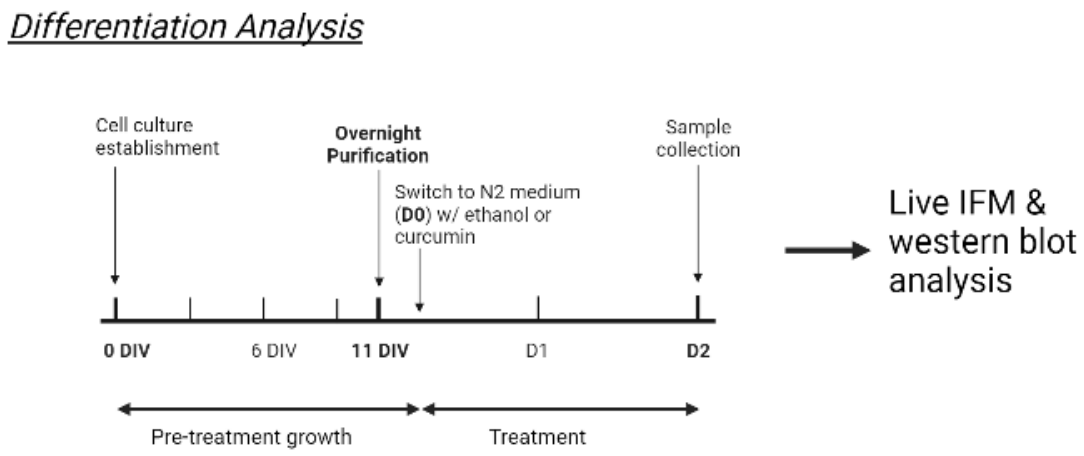

Figure 3: The experimental design for the differentiation analysis in enriched OPC cultures *via* live IFM and western blotting. Created with BioRender.com.

Analysis of Protective Effects

The enriched OPC cultures that were seeded on 96-well plates from the sample set 2 were fed with 5% FBS for 11 DIV. On 11 DIV, the 5% FBS medium was switched to a chemically defined serum-free N2 medium containing ethanol as a control vehicle or 1 µM curcumin. The treatment was continued until Day 2 (D2) of differentiation (48-hour treatment).

Following the 48-hour treatment, the cells were challenged with thapsigargin for 3 hours. The YO-PRO immunofluorescent dye was used to obtain cell absorbance data based on the plat reader method (Figure 4).

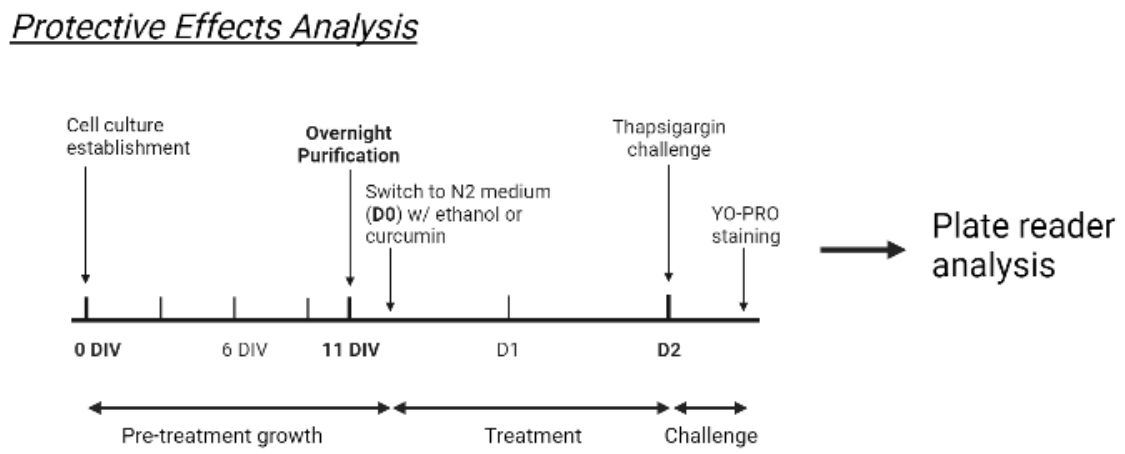

Figure 4: The experimental design for the analysis of curcumin's protective effects in enriched OPC cultures *via* the plate reader method. Created with BioRender.com.

Signaling Analysis

The enriched OPC cultures that were seeded on 6-well plates from the sample set 2 were treated for 1 hour prior to sample collection. The OPC cultures were fed with 5% FBS for 11 DIV. On 11 DIV, the 5% FBS medium was switched to insulin-free N2 medium. On Day 2 of differentiation, 1 hour prior to sample collection, the OPC cultures were treated with N2 medium containing ethanol as a control vehicle or 1 µM curcumin treatment. Following the 1-hour treatment, the OPC cultures were collected and stored at –80˚C for further western blot analysis (Figure 5).

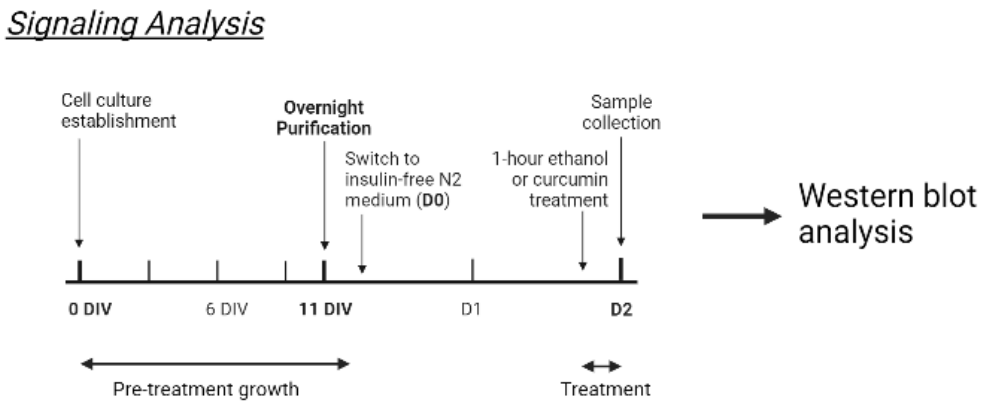

Figure 5: The experimental design for the signaling analysis in enriched OPC cultures *via* western blotting. Created with BioRender.com.

Experimental Analysis Techniques

Live Immunofluorescence Microscopy (IFM)

Immunofluorescence microscopy was performed following a previously discussed protocol to evaluate cell differentiation based on oligodendrocyte surface antigens (Guardiola-Diaz et al. 2012). The samples were incubated in primary antibody solution, including O4 (1:25) and anti-HPC7 (1:40) in 3% NGS/HE for 15 minutes on ice. The cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT). The samples were incubated in secondary antibody solution, including Hoechst nuclear label dye (1:200, Sigma); FITC-conjugated goat anti-mouse IgM, μ-chain specific, for O4 (1:50); Cy2-conjugated anti-mouse IgG, gamma-chain specific for HPC7 (1:600) in 3% NGS/HE. The incubation continued for 20 minutes at RT in the dark. Coverslips were fixed by using 1 drop of DABCO solution and the samples were analyzed by using an Olympus fluorescence

microscope. Randomly chosen 10 consecutive fields per well were counted and averaged for further data analysis (Guardiola-Diaz et al. 2012).

Western Blot Analysis.

Cell signaling was evaluated by western blot analysis. Samples were lysed in RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% NP40 and 1% TX-100, pH 7.4), including phosphatase and protease inhibitors (1mM PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 1 mM orthovanadate, fluoride 50 mM, pyrophosphate 10 mM) and centrifuged at 15,000 g for 15 min at 4 °C. Proteins were separated by electroporation on 10-20% Tris-Glycine gels and transferred to nitrocellulose membranes. Membranes were blocked with blocking buffer (Tris buffered saline with 0.1% Tween 20 and 5% w/v nonfat dry milk). The membranes were incubated with primary antibodies GAPDH (1:1000, Cell Signaling Technologies) and CNPase (1:1000, Cell Signaling Technologies) overnight. The membranes were washed and incubated with secondary Anti-Rabbit IgG HRP-linked antibody (1:2000, Cell Signaling Technology) for 1 hour, followed by protein detection with luminol-based ECL western blotting substrate (Cell Signaling Technology, 2005).

Plate Reader Analysis

The plate reader analysis method was derived from a previously discussed procedure (Rat et al. 2017). After treating samples from 96-well plates with N2 medium with ethanol as a control vehicle or 1 µM curcumin starting from 11 DIV (Day 0 of differentiation), the treatment was continued until Day 2 (D2) of differentiation. On D2 of differentiation, the samples were challenged with thapsigargin treatment (100 nM) for 3 hours. Following the 3-hour treatment with thapsigargin, the samples were treated with YO-PRO nucleic acid stain

for 15 minutes. After the YO-PRO treatment, the OPC absorbances were measured through the plate reader method to quantify apoptotic cells in the samples.

Statistical Analysis

Data was analyzed and shown as means ± SEM. One-way ANOVA was used for statistical analysis of the data (Microsoft Excel Office 365 and confirmed by online resource http://vassarstats.net/). The accepted statistically significant *p*-value was $p < 0.05$.

Results

The effect of curcumin on oligodendrocyte differentiation was evaluated in two distinct cell populations – mixed glial cultures and OPC cultures – to account for the potential effect of curcumin on differentiation in two different contexts. The use of mixed glial cultures allowed consideration of the heterogeneous cellular composition of the CNS (McCarthy & de Vellis, 1980). This method created a physiologically relevant study model for oligodendrocyte differentiation as it accounts for the interactions between oligodendrocytes and other glial cells (such as astrocytes) (McCarthy & de Vellis, 1980). Nevertheless, the use of mixed glial cultures also presented a limitation to the study as this heterogeneous composition may interfere with the identification of specific biochemical characteristics of distinct cell populations and their individual differentiation mechanisms (McCarthy & de Vellis, 1980). The use of OPC cultures, consistent with previous research designs, allowed for studying the molecular mechanisms and pathways directly involved in OPC culture differentiation *in vitro* in the context of myelination (Yang et al. 2016).

Curcumin treatment shows a significant decrease in the average total nuclei number in mixed glial cultures

In this study, we wanted to evaluate if curcumin has any anti-proliferative or toxic effects on oligodendrocytes based on the total cell counts. Mixed glial cultures were treated with ethanol (control vehicle), 1 µM curcumin, or 5 µM curcumin for 2 days *in vitro* (DIV). Following treatment, the cultures were stained with Hoechst dye for their nuclei counts and for O4 antigenic marker as a part of the live IFM protocol. O4 is a cell surface marker that is expressed in response to the transition of OPCs to the late progenitor differentiation stage and is indicative of the loss of the bipolar morphology of the cells (Kuhn et al. 2019). The results from Hoechst dye staining of the nuclei showed a significant difference in the average nuclei counts between the control and curcumin treatments ($p<0.05$, Figure 6). Further statistical analysis *via* the Tukey HSD test (http://vassarstats.net/) showed a significantly lower value for the average number of nuclei in 5 µM mixed glial culture treatment compared to the control treatment ($p<0.05$, Figure 6). There was no statistical significance seen between the control group and the 1 µM treatment group or between the 1 µM treatment group and the 5 µM treatment group ($p>0.05$, Figure 6). Nevertheless, the results showed a decreasing trend in the average number of nuclei with the addition of curcumin in a concentration-dependent fashion in mixed glial cultures.

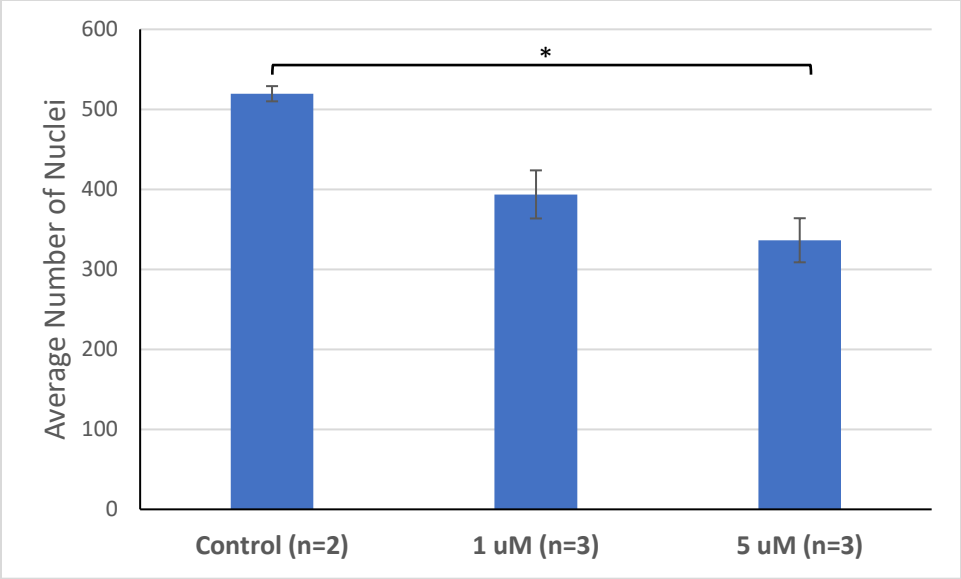

Figure 6: Curcumin effects on the average nuclei counts in mixed glial cultures. The effects of a 2-day treatment with 1 µM and 5 µM curcumin were examined *via* live IFM. The average nuclei counts were conducted based on Hoechst dye staining and presented as mean ± SEM. The control and 5 µM curcumin treatment showed a significant difference in their average nuclei counts *p<0.05

Curcumin treatment shows no significant effect on oligodendrocyte differentiation in mixed glial cultures

We wanted to further evaluate if curcumin treatment has differentiation-enhancing effects on oligodendrocyte cells in mixed glial cultures. The live IFM data analysis for O4 antigenic marker staining was used to generate a comparison between immature and committed cells. The ratio between the total committed cell counts over the total O4-positive cell counts was calculated as a percentage for each experimental group. The results showed no significant differentiation effects of curcumin on oligodendrocyte progenitor cells in mixed glial cultures ($p>0.05$, Figure 7). There was no data collected for the 5 µM curcumin treatment condition due to defective staining or possible substantial cell death. Nevertheless, there is a noticeable difference observed between the control and the 1µM curcumin treatment in OPC cultures, as

there is a decreased ratio between the total committed cell counts over the total O4-positive cell counts with the addition of 1µM curcumin compared to the control. While the error bars are large due to the low sample size used in the experiment, there is still a noticeable effect of curcumin on oligodendrocyte differentiation in mixed glial cultures. Interestingly, there are no substantial differences seen between the two control samples. Thus, the large error bars are not indicative of the variability between samples but may have been caused due to the calculation methods used in the experiment that evaluate the relative ratio for differentiation. Future studies can eliminate such limitations of the experimental design by increasing the sample size for each treatment group to be able to reach clearer conclusions based on the data obtained.

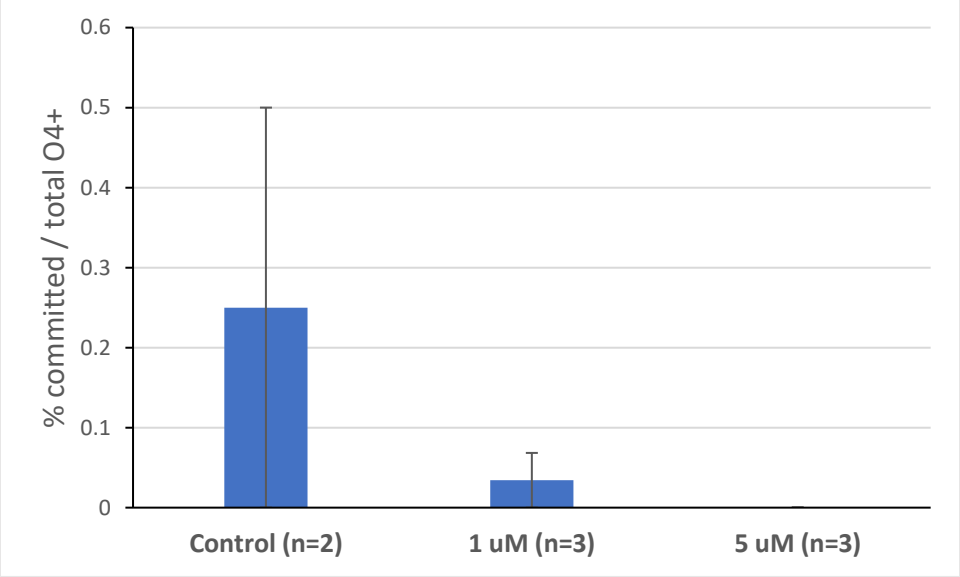

Figure 7: Curcumin effects on the differentiation of oligodendrocyte progenitor cells in mixed glial cultures. The effects of a 2-day treatment with 1 µM and 5 µM curcumin were examined *via* live IFM. The cell counts were conducted based on O4 staining. The percentage of committed cells was evaluated based on their ratio compared to total O4+ cells that include immature and committed oligodendrocytes. The obtained values were presented as mean ± SEM. No significant difference between treatments was seen ($p>0.05$).

Curcumin treatment shows no effect on the average total nuclei number in oligodendrocyte progenitor cell (OPC) cultures

We wanted to further assess if the decrease in the average total nuclei number following curcumin treatment in mixed glial cultures can be replicated in OPC cultures. Two sets of experiments were conducted on two separately obtained samples of OPC cultures and evaluated independently to consider the potential differences and variability between the obtained cultures. Both experiments followed the same protocol and experimental design. (OPC cultures were treated with ethanol (control vehicle), 1 μM curcumin, or 5 μM curcumin for 2 DIV. Due to limited samples and the potential toxicity of 5 μM curcumin treatment, this treatment group was eliminated for the sample set 1 for the OPC culture study.) Following curcumin treatment, the cultures were stained with Hoechst dye for their nuclei counts, and for the O4 and HPC7 antigenic markers to evaluate the differentiation-mediation properties of curcumin.

The results from Hoechst dye staining of the nuclei for the sample set 1 showed no significant difference in the average nuclei counts between the control and 1 μM curcumin treatment ($p>0.05$, Figure 8). Similarly, the results from Hoechst dye staining of the nuclei for the sample set 2 showed no significant difference in the average nuclei counts between the control and 1 μM curcumin treatment ($p>0.05$, Figure 9). While statistically insignificant, sample set 1 showed an increase in the average total nuclei counts following 1 μM curcumin treatment which was inconsistent with the results obtained for the mixed glial cultures. However, sample set 1 comprised an extremely small sample size with only one control group and three treatment groups. In contrast, sample set 2 showed a similar decreasing trend following 1 μM curcumin treatment as seen in mixed glial cultures. Nevertheless, sample set

2 showed an increasing average number of nuclei following 5 µM curcumin treatment, which was inconsistent with the results obtained for mixed glial cultures. This inconsistency could be a result of having only one sample for the 5 µM curcumin treatment, which may not fully represent the effects of curcumin treatment on total nuclei numbers.

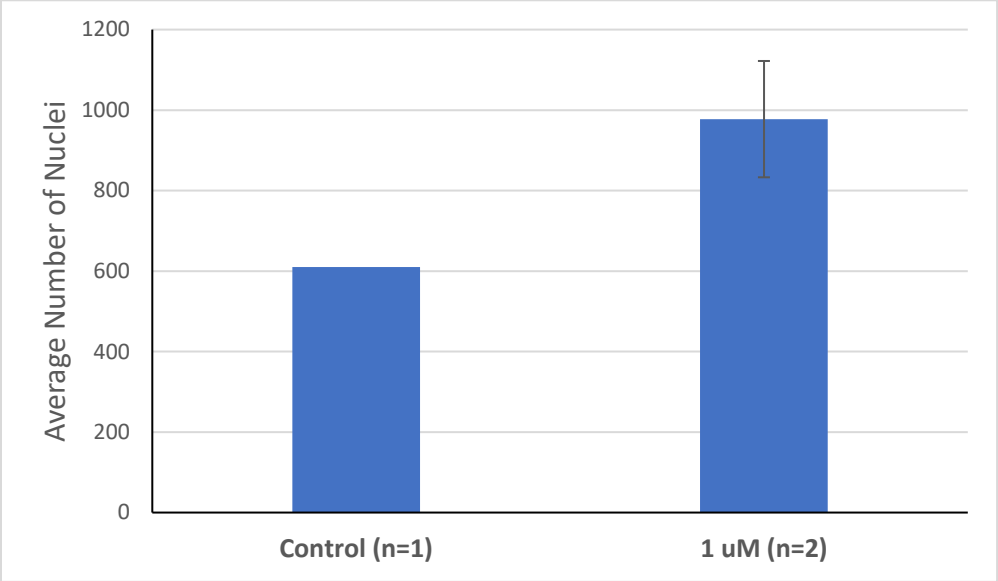

Figure 8: Curcumin effects on the average nuclei counts in enriched OPC cultures in the sample set 1. The effects of a 2-day treatment with 1 µM curcumin were examined *via* live IFM. The average nuclei counts were conducted based on Hoechst dye staining and presented as mean ± SEM. No significant difference between treatments was seen ($p>0.05$).

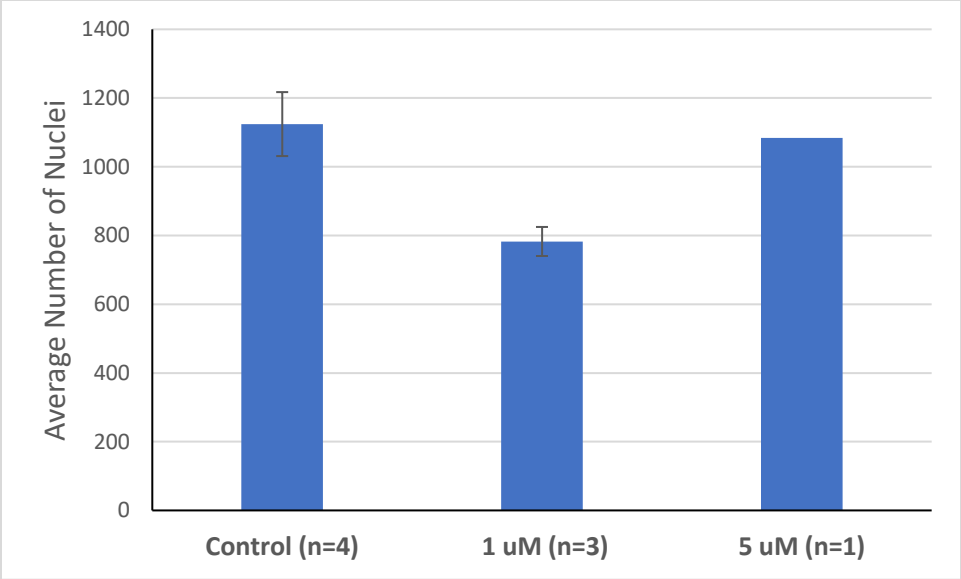

Figure 9: Curcumin effects on the average nuclei counts in enriched OPC cultures in the sample set 2. The effects of a 2-day treatment with 1 µM curcumin were examined *via* live IFM. The average nuclei counts were conducted based on Hoechst dye staining and presented as mean ± SEM. No significant difference between treatments was seen ($p>0.05$). The decrease in the average number of nuclei as a result of 1 µM curcumin treatment was consistent with the results obtained in mixed glial cultures.

Curcumin shows a significant effect on oligodendrocyte differentiation in OPC cultures based on the antigenic marker and morphological analyses

To evaluate the effect of curcumin on OPC differentiation *in vitro*, OPC cultures were assessed based on their morphology and stage-specific antigenic markers with or without curcumin treatment. Early purified OPCs were treated with ethanol as a control vehicle and with 1 µM curcumin for 2 days after purification for both sample set 1 and sample set 2. All nuclei were stained with Hoechst dye and counted for the total number of cells present. The treated samples were immunolabeled with markers for O4 and HPC7. Morphology evaluation was done based on previously identified stage-specific morphological and branching characteristics of isolated oligodendrocytes from the rat brain telencephalon (Guardiola-Diaz et al. 2012). Early progenitors – the first identifiable morphological

structures – have the simplest morphological characteristics with no specific branching, and can be identified through antigenic markers Olig2 and A2B5 (Guardiola-Diaz et al. 2012). The OPC cultures in our experiment were not immunolabeled with Olig2 and A2B5 as we did not expect to see early progenitors after 48-hour development and differentiation of the cells. Their transition to the late progenitor stage is associated with slight branching and the expression of the O4 antigenic marker (Guardiola-Diaz et al. 2012). Late progenitors enter a sub-stage of differentiation through their expression of GalC, which marks their commitment to terminal differentiation and their further branching into immature oligodendrocytes (Guardiola-Diaz et al. 2012). Immature oligodendrocytes can be additionally recognized by their expression of the HPC7 marker in later immature oligodendrocyte differentiation stages (Guardiola-Diaz et al. 2012). Fully mature oligodendrocytes, with extensively branched morphology, show a circular branched structure, and the expression of MBP and MOG markers that are indicative of mature oligodendrocytes (Guardiola-Diaz et al. 2012).

The ratio between the total committed cell counts over the total O4-positive cell counts was calculated as a percentage for each experimental group of each sample set. The results for the sample set 1 showed no significant differentiation-enhancing effects of curcumin on oligodendrocyte cells in OPC cultures evaluated based on morphology in O4-positive cells ($p>0.05$, Figure 10). The ratio between the HPC7-positive cells over the total O4-positive cell counts based on HPC7 immunoreactivity was calculated as a percentage for each experimental group. The results for the sample set 1 showed no significant differentiation-enhancing effects of curcumin on oligodendrocyte cells in OPC cultures based on HPC7 immunoreactivity ($p>0.05$, Figure 11). While insignificant, the results show a trending increase in differentiation in OPC cultures following curcumin treatment based on both the

morphology of O4-positive cells and their HPC7 immunoreactivity. These results are inconsistent with the analysis of the effects of curcumin on differentiation in mixed glial cultures. As previously identified, the experimental study for oligodendrocyte differentiation had a small sample size. Similarly, sample set 1 had only one control and two curcumin treatment samples, thus comprising a limited data set for the analysis. Consequently, it was important to evaluate the same experimental procedure on sample set 2 which had a higher number of samples for each variable. Additionally, sample set 2 contained enough experimental samples to add a 5 µM curcumin treatment to the experiment, which was eliminated from the experimental setup for the sample set 1 due to limited sample size.

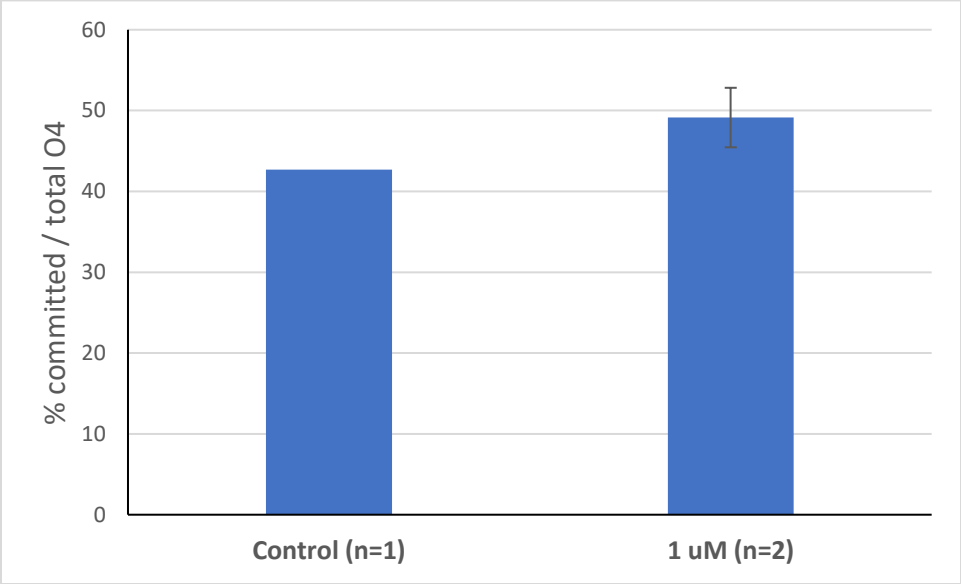

Figure 10: Curcumin effects on oligodendrocyte differentiation in enriched OPC cultures in the sample set 1. The effects of 48-hour treatment with 1 µM curcumin treatment were examined *via* live IFM. The percentage of committed oligodendrocytes relative to the total O4+ cells was calculated based on morphology in O4+ cells. The data were presented as mean ± SEM. No significant difference between the percentage of committed oligodendrocytes to the total O4+ cells for control and 1 µM curcumin treatments was seen ($p>0.05$). There is an increasing trend of differentiation enhancement seen in response to 1 µM curcumin treatment.

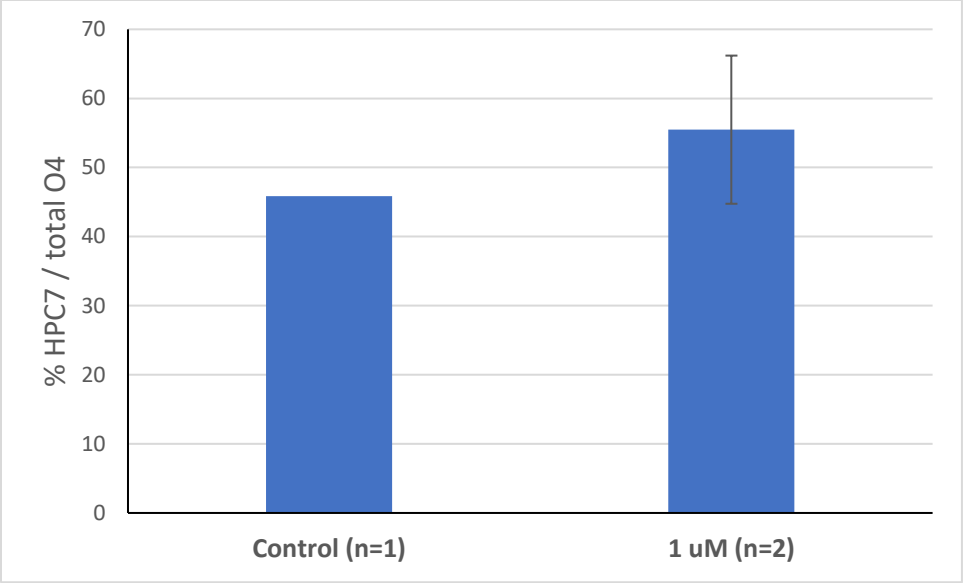

Figure 11: Curcumin effects on oligodendrocyte differentiation in enriched OPC cultures in the sample set 1. The effects of 48-hour treatment with 1 µM curcumin treatment were examined *via* live IFM. The percentage of HPC7+ cells relative to the total O4+ cells was calculated based on HPC7 immunoreactivity. The data were presented as mean ± SEM. No significant difference between the percentage of HPC7+ oligodendrocytes to the total O4+ cells for control and 1 µM curcumin treatments was seen ($p>0.05$). There is an increasing trend of differentiation enhancement seen in response to 1 µM curcumin treatment.

The results for the sample set 2 showed significant differentiation-enhancing effects of curcumin on oligodendrocyte cells in OPC cultures based on morphology in O4-positive cells compared to the control ($p<0.05$, Figure 12). Additionally, the results for the sample set 2 showed significant differentiation enhancing effects of curcumin on oligodendrocyte cells in OPC cultures compared to the control based on HPC7 immunoreactivity ($p<0.05$, Figure 13). Nevertheless, the differentiation-enhancing effect of curcumin did not show concentration dependence. While inconsistent with the results obtained from mixed glial cultures, these results are consistent with the differentiation-enhancing trend seen in sample set 1.

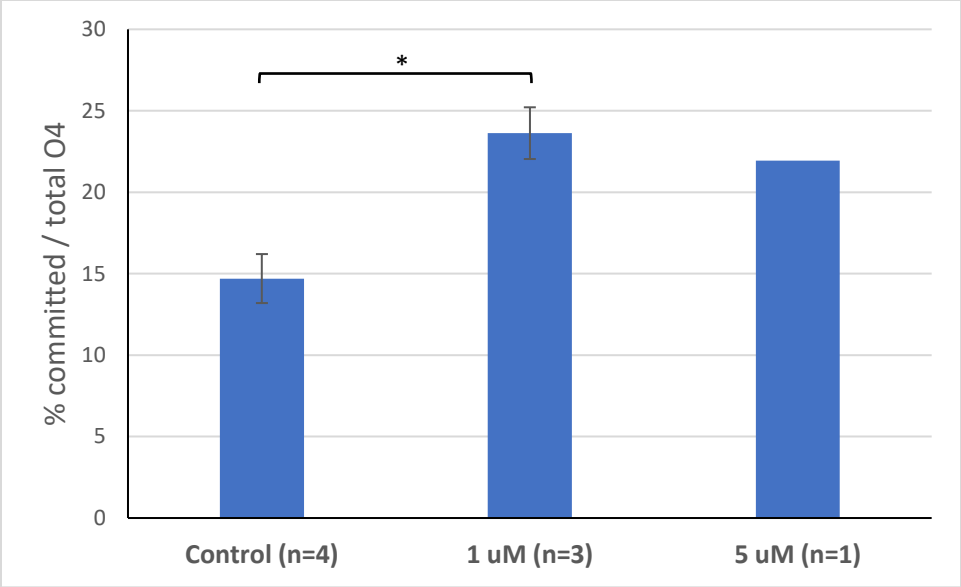

Figure 12: Curcumin effects on oligodendrocyte differentiation in enriched OPC cultures in the sample set 2. The effects of 48-hour treatment with 1 µM curcumin treatment were examined *via* live IFM. The percentage of committed oligodendrocytes relative to the total O4+ cells was calculated based on morphology in O4+ cells. The data were presented as mean ± SEM. The results show a significant difference between the percentage of committed oligodendrocytes to the total O4+ cells for control and 1 µM curcumin treatments *p<0.05.

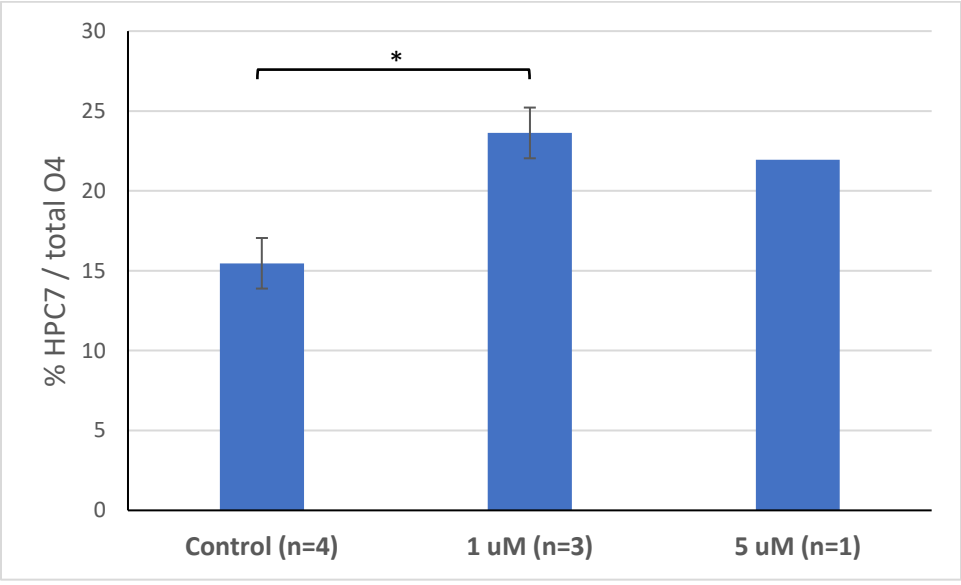

Figure 13: Curcumin effects on oligodendrocyte differentiation in enriched OPC cultures in the sample set 2. The effects of 48-hour treatment with 1 µM curcumin treatment were examined *via* live IFM. The percentage of HPC7+ cells relative to the total O4+ cells was calculated based on HPC7 immunoreactivity. The data were presented as mean ± SEM. The results show a significant difference between the percentage of HPC7+ oligodendrocytes to the total O4+ cells for control and 1 µM curcumin treatments *p<0.05.

Curcumin's protective abilities remain unresolved pending the development of new methods for challenging OPC cultures and detecting OPC apoptosis

We wanted to further evaluate if curcumin shows any possible protective properties in case of a challenge presented to the OPC cultures. Thapsigargin was the compound of choice to present a challenge to the OPC cultures. Thapsigargin is a commonly used endoplasmic reticulum (ER) stressor that causes decreased Ca^{2+} levels and unfolded protein response (UPR) by the inhibition of sarco/endoplasmic reticulum Ca^{2+}-ATPase (Lindner et al. 2020). OPC cultures were treated with either ethanol (control vehicle), thapsigargin (100 nM) and ethanol, 1 μM curcumin, or 1 μM curcumin and thapsigargin. The ethanol and curcumin treatments were done for 2 DIV. Thapsigargin was added after 2 DIV for a 3-hour treatment. YO-PRO nucleic acid stain was used as an immunofluorescent dye to fluorescently label apoptotic cells (Rat et al. 2017). Following the addition of the YO-PRO nucleic acid stain, the OPC absorbances were measured through the plate reader method to measure the extent of apoptosis after each treatment. The results showed no significant differences between treatment groups ($p>0.05$, Figure 14). Interestingly, there was no significant difference seen between the control and the thapsigargin treatment group ($p>0.05$, Figure 14). Thus, this insignificance indicates that the experimental design did not accomplish a necessary level of a challenge through ER stress induction to evaluate the possible protective effects of curcumin in oligodendrocytes. These results also suggest the need to develop a concrete methodology to present a challenge to OPC cultures in an experimental setting and expand the assessment of curcumin's potential protective properties in response to a challenge.

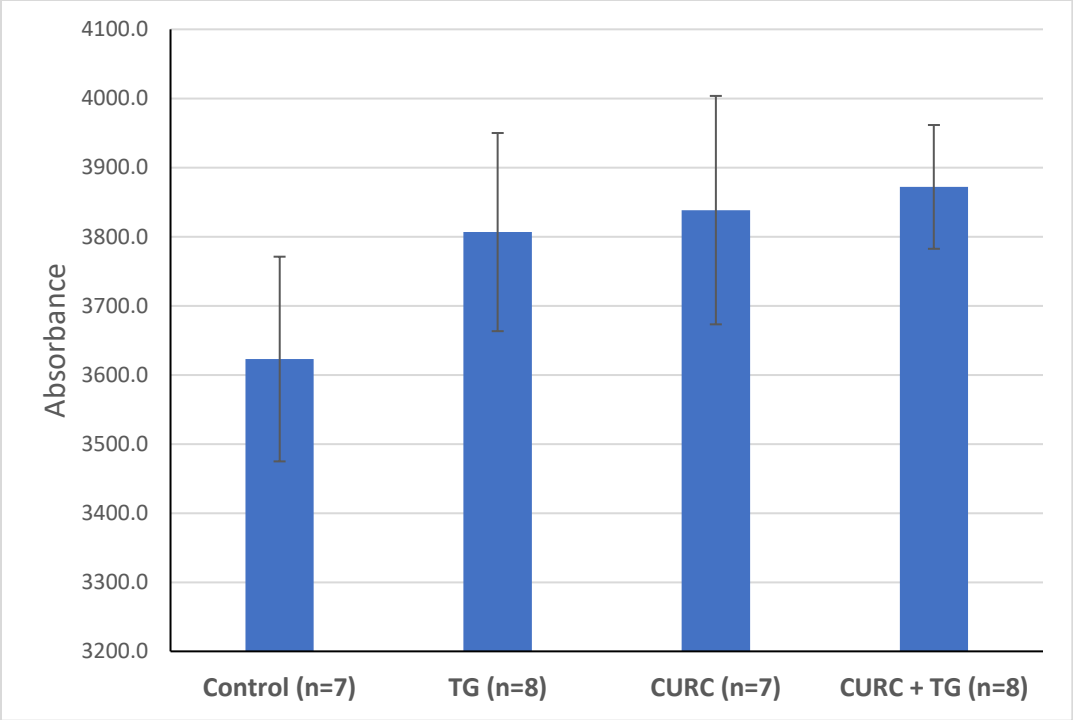

Figure 14: Curcumin activity against a challenge in OPC cultures. The protective effect of curcumin was examined by the addition of thapsigargin (100 nM) for 3 hours following a 48-hour treatment with 1 µM curcumin. Cellular apoptosis of OPC treatment groups was evaluated through the plate reader method using YO-PRO nucleic acid stain. The data were presented as mean ± SEM. No significant difference between treatment groups was seen ($p>0.05$).

Curcumin's effects on signaling pathways involved in oligodendrocyte differentiation and development remain undetermined until further study conduction

We wanted to further assess if curcumin's differentiation-enhancing abilities are associated with the signaling pathways involved in oligodendrocyte differentiation and development. We aimed to focus on the Ras/Raf/Mek/Erk and PI3K/Akt/mTOR signaling pathways that are involved in distinct steps of oligodendrocyte differentiation (Guardiola-Diaz et al. 2012). Particularly, we aimed to focus on the Akt/mTOR activity due to its impact on myelination as Akt/mTORC1 inhibition has been shown to cause reduced myelination (Grier et al. 2017).

Previous studies have demonstrated the role of insulin in the activation of Akt and mTOR protein kinases for cellular development (Vander Haar et al. 2007). To account for this effect, we used insulin-free N2 media for the signaling analysis procedure to silence signaling before the stimulation of OPC cultures with 1 μM curcumin. Our initial experiment used GAPDH and 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) primary antibodies. The "housekeeping" gene GAPDH was used as a control to evaluate the relative gene expression of other genes as commonly seen in previous literature (Barber et al. 2005). CNPase is a known marker associated with myelin expression that is activated in later stages of oligodendrocyte lineage progression as OPCs start to lose their bipolar morphology and extend their myelinating processes (Grier et al. 2017; Kuhn et al. 2019). Unfortunately, we were unable to obtain data from the signaling analysis study due to complications related to the protein concentrations in collected samples and inconsistent GAPDH expression for the western blotting procedure. Additionally, we were unable to conduct further experiments to quantify MBP expression in response to curcumin treatment due to limited samples, as well as expand our experiments on studying the role of specific signaling pathways in curcumin activity. Future studies should aim to repeat these experimental procedures with larger sample sets, as well as focus on identifying if curcumin's differentiation-enhancing activity is associated with its regulation of the Ras/Raf/Mek/Erk and/or PI3K/Akt/mTOR signaling pathways.

Discussion

This study aimed to evaluate the effect of curcumin on oligodendrocyte differentiation in two distinct cultures – mixed glial cultures and oligodendrocyte progenitor cultures. Additionally, it aimed to identify potential cytotoxic or anti-proliferative effects of curcumin that may influence the total cell counts in the cultures. Through a pilot experiment, the study also aimed to further explore the effects that curcumin pre-treatment has in response to a challenge in oligodendrocytes and identify its potential protective effects following a challenge. The broader implication of the study findings aimed to enhance our understanding of the potential use of curcumin as a therapeutic treatment for multiple sclerosis (MS). Through biochemical and morphological analyses, we evaluated the effects of curcumin on average cell numbers and oligodendrocyte differentiation in two distinct cell cultures, and the potential protective effects of curcumin against a challenge in OPC cultures.

Our study results showed no significant effects of curcumin on oligodendrocyte differentiation in mixed glial cultures. There was a noticeable trend of decreasing oligodendrocyte differentiation in response to curcumin addition in mixed glial cultures based on O4 antigenic marker evaluation. In contrast, OPC cultures showed a differentiation-enhancing trend for both sample sets in response to curcumin treatment with significant results for 1 μM curcumin treatment for the sample set 2. This evaluation was based on antigenic marker staining of O4 and HPC7 that allowed both morphological and biochemical assessment of oligodendrocyte differentiation. These markers were chosen due to their previously identified role in specific differentiation stages of oligodendrocytes and our predictions for the differentiation stages that we should focus on for a 48-hour study (Guardiola-Diaz et al. 2011). These results supported our hypothesis that predicted

differentiation-enhancing properties of curcumin treatment. These findings are also consistent with previous literature that demonstrates curcumin's ability to enhance oligodendrocyte differentiation (Bernardo et al. 2021; Dikmen, 2017). Additionally, these findings support previously identified *in vivo* and *in vitro* effects of curcumin in the context of multiple sclerosis therapy (Ghanaatian et al. 2018; Xie et al. 2011).

To our knowledge, there are no *in vitro* studies assessing the effect of curcumin treatment on oligodendrocyte differentiation in mixed glial cultures. Most studies done on oligodendrocyte differentiation focus on homogenous cell culture isolation and subsequent treatment of the culture in an isolated environment (Beasley, 2015). However, the isolated homogenous environment does not replicate the heterogeneous *in vivo* conditions (Beasley, 2015). A study conducted by Beasley (2015) attempted to identify the co-dependence between oligodendrocytes and astrocytes in heterogeneous mixed glial cultures. The study found that astrocytes and oligodendrocytes are dependent on each other due to the production of growth factors by each cell that supports the other's growth and healthy maturation in a heterogenous culture (Beasley, 2015). Nevertheless, the study showed that it is possible to independently target oligodendrocytes in mixed glial cultures without interfering with the astrocyte functionality (Beasley, 2015). Following the findings of Beasley (2015), it can be suggested that our live IFM study conducted in mixed glial cultures represents the differentiation-based effects of curcumin on oligodendrocytes without affecting astrocytes. However, the study by Beasley (2015) was not able to confirm that targeted astrocyte death has no effect on oligodendrocyte functionality and growth. An additional study has identified a separate mechanism by which OPCs differentiate into astrocytes (type II astrocytes) rather than oligodendrocytes in mice depending on culture conditions and media used (Suzuki et al.

2017). In our study, if curcumin has any adverse effects on astrocytes in mixed glial cultures, these negative effects could be responsible for astrocyte death and subsequent decrease in OPC numbers. This reasoning may explain the reduction in average total nuclei number and decreased differentiation of oligodendrocytes in mixed glial cultures that were inconsistent with our results in OPC cultures. Alternatively, the decrease in average nuclei number and differentiation could be the result of OPC differentiation into type II astrocytes, thus reducing the number of OPCs and the extent of OPC differentiation into oligodendrocytes. Further studies are needed to identify if curcumin affects astrocyte functionality and has potential apoptosis-causing activity in astrocytes, and if astrocyte death can influence the growth and differentiation of OPCs. Additionally, further studies conducted in mixed glial cultures can attempt to immunolabel and quantify both astrocyte and oligodendrocyte numbers to assess if there is an increase in astrocyte numbers due to type II astrocytes.

Previous research has identified the limitations of curcumin due to its biochemical structure and dose-dependent toxicity (Mohajeri et al. 2015). Curcumin shows toxicity and apoptotic activity in 5 μM concentration *in vitro* in purified enriched oligodendrocyte progenitor cell cultures (Bernardo et al. 2021). Curcumin's apoptotic activity and toxicity can be another possible reason behind our findings of significantly lower total average nuclei counts in 5 μM curcumin-treated mixed glial cultures compared to the control. Conversely, these results can be indicative of the interplay between differentiation and proliferation. Cell differentiation and proliferation are known to have an inverse relationship as proliferation decreases with further enhancement of differentiation (Ruijtenberg & van den Heuvel, 2016). As a result, the lower total cell counts in curcumin-treated mixed glial cultures may be a potential indicator of differentiation enhancing and anti-proliferative properties of curcumin,

rather than of a cytotoxic effect. Further studies should explore both hypothesized effects to distinguish between them. These studies can utilize the Tunel assay to assess the extent of the apoptotic effect that curcumin has on oligodendrocytes and if this effect shows a concentration-dependent mechanism. The Tunel assay has been used in a prior study with OPC cultures that suggested the apoptotic activity of curcumin in 5 μM concentration (Bernardo et al. 2021). However, to our knowledge, there have been no such studies done in mixed glial cultures or OPC cultures with different curcumin concentrations (Bernardo et al. 2021). To assess if there is any anti-proliferative activity following curcumin treatment, CyQuant and MTT assays can be utilized as OPC proliferation assays that have also been previously used in literature (Becker-Catania et al. 2011; Bernardo et al. 2021). These additional assays could distinguish between the apoptotic and anti-proliferative activity of curcumin. Additional experiments can also conduct a titration study to identify the most optimal concentration of curcumin based on its effectivity, as well as the concentrations that cause severe cell death in cultures.

Recent studies on curcumin have focused on targeting its solubility limitations by making biochemical and structural modifications to the original compound composition (Naeimi et al. 2018; Mohajeri et al. 2015). Mohajeri et al. (2015) showed the higher effectivity of polymerized nano-curcumin (PNC) to enhance myelination in *in vivo* and *in vitro* contexts. The study showed that PNC treatment of OPCs increases the gene expression of Nestin as an antigenic marker for neural stem cells, Olig2 and PDGFRα as markers for OPCs, and MBP as an indicator of myelinating oligodendrocytes (Mohajeri et al. 2015). The authors suggested that these results can be indicative of PNC's ability to cause neural stem cell differentiation into OPCs and subsequent OPC differentiation to their mature forms in

EAE-induced mice (Mohajeri et al. 2015). Additionally, PNC showed significantly higher effectivity on alleviating EAE clinical symptoms compared to curcumin, thus further indicating its higher effectivity in the MS context compared to curcumin (Mohajeri et al. 2015). Further research should aim to identify the comparative effects of curcumin and PNC on oligodendrocyte differentiation and development in both OPC cultures and mixed glial cultures.

As a part of the study, we conducted a pilot experiment to evaluate the potential protective effects of curcumin on OPCs in response to a challenge or cell damage. Thapsigargin has been previously identified as an endoplasmic reticulum stressor in oligodendrocytes, used particularly in MS research (Chen et al. 2019). Due to previously obtained data in our laboratory, thapsigargin treatment for this study was limited to 3 hours in 100 nM concentration. Nevertheless, a previous study by Chen et al. (2019) presented the use of thapsigargin (200 nM) in oligodendrocytes for up to 8 hours. As seen in our findings, thapsigargin treatment (100 nM) for 3 hours did not cause any adverse effects in OPC cultures *in vitro*. The ineffectiveness of the thapsigargin treatment could be a result of its lower final concentration or shorter treatment span used in our study. Further studies should aim to identify the optimal standards for thapsigargin treatment in OPC cultures, including the time of the treatment and its final concentration. Following the identification of optimal thapsigargin treatment standards, future studies should focus on utilizing thapsigargin treatment as a method to study the potential protective effects of curcumin in OPC cultures. Additionally, the pilot experiment used a protocol developed by a previous research study to evaluate the effect of thapsigargin and curcumin treatment on OPC cultures via YO-PRO nucleic acid staining and a 96-well plate-based analysis method (Rat et al. 2017). Based on

our findings, we successfully used the plate reader method for OPC cultures and obtained absorbance values from YO-PRO nucleic acid staining. Nevertheless, future studies should further explore the combinatorial use and the alteration of the thapsigargin treatment and the plate reader methods to develop an efficient procedure for OPC culture damage and subsequent evaluation of curcumin's potential protective effects in oligodendrocytes.

The present study shows the importance of continuing further research to identify the role of curcumin in oligodendrocyte differentiation in the context of MS. These studies are necessary to explore curcumin's activity in the context of oligodendrocyte differentiation *in vivo* and *in vitro* and to confirm previously obtained findings through larger-scale studies. The main limitations of our study are small sample sizes and the limited availability of literature for the development of the experimental design. Future studies should aim to utilize a larger-scale analysis in *in vivo* and *in vitro* models. Moreover, an important addition to the study of curcumin's role in oligodendrocyte differentiation would be a large-scale titration curve to identify the most optimal curcumin concentrations to be used in further experimental procedures. Following these investigations, research studies should focus on identifying the biochemical mechanisms involved in the differentiation-enhancing properties of curcumin, including but not limited to the identification of the roles of Ras/Raf/Mek/Erk and PI3K/Akt/mTOR pathways in curcumin activity. These future studies focused on identifying the differentiation-enhancing effects of curcumin can help us to move a step forward in involving natural products, such as curcumin, in the development of therapeutic options for MS and other neurodegenerative and autoimmune diseases.

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