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Original Article

Cannabidiol Drives Efficient Neural Differentiation of Human Wharton's Jelly Mesenchymal Stem Cells: A Small-Molecule Approach for *In Vitro* Neurogenesis

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Abstract

Introduction: Over the past decade, research on small molecules such as cannabidiol (CBD) has expanded due to their ability to modulate biological pathways through protein interactions. Understanding how mesenchymal stem cells (MSCs) differentiate into neural-like cells is crucial for developing effective therapies for neurological disorders. This study aimed to investigate the efficacy of cannabidiol, as a cost-effective small-molecule inducer, in promoting the neural differentiation of human Wharton's jelly MSCs (hWJ-MSCs) *in vitro* and to determine the optimal non-cytotoxic dose for this purpose.

Materials and Methods: The optimal CBD dose (5 µg/ml) was first determined using the MTT assay and acridine orange/ethidium bromide (AO/EB) staining. To evaluate neural differentiation potential, the expression of neural marker genes, MAP-2, *NSE*, *Oligo-2*, *β-tubulin III*, and *GFAP*, was assessed by real-time PCR at 7 and 14 days' post-induction.

Results: The results demonstrated significant upregulation of neural marker genes at 7 and 14 days after CBD treatment, confirming successful induction of neural differentiation.

Conclusions: The designed protocol is highly effective and efficient for inducing neural differentiation. Further *in vivo* studies using biocompatible scaffolds are necessary to elucidate the underlying mechanisms and therapeutic implications of these findings.

Keywords: Cannabidiol, Small Molecules, Wharton's Jelly Mesenchymal Stem Cells, Neural-Like Cells

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Introduction

Neurological disorders, along with cardiovascular diseases, represent a major global health challenge, affecting billions of people and imposing a significant economic burden on healthcare systems.[1,2] Neurodegenerative diseases gradually worsen over time and pose a serious threat to human health. According to a report by the World Health Organization (WHO), neurodegenerative diseases are projected to become the second leading cause of death worldwide by 2040, following cancer. Given the limited regenerative capacity of the human nervous system, the progression of these diseases is considered largely irreversible.[3,4]

Repair of nervous system damage is an extremely slow process. It may take months or even years to reestablish functional connections, and in some cases, this may never occur. Damaged neurons often enter a state known as chronic axotomy.[5,6] Given the importance of the nervous system and its key role in the body's physiology, numerous studies are underway worldwide to address the challenges of effective nervous system repair and functional recovery, often focusing on novel therapeutic strategies.

The use of cell therapy methods dates back to the 19th century. Cell therapy includes stem cell-based and non-stem cell-based treatments, encompassing both single-cell and multi-cell therapies with various immunophenotypic profiles, isolation techniques, mechanisms of action, and regulatory levels.[7] Stem cell therapy is a subset of cell therapy that has

recently garnered significant attention. In this therapeutic approach, stem cells, particularly mesenchymal stem cells (MSCs), which possess a high capacity for differentiation into various cell types, are used to treat various diseases.[8,9]

MSCs are multipotent adult stem cells capable of self-renewal and differentiation into multiple cell lineages.[10] Owing to their unique properties, these cells are considered promising candidates for targeted therapies, especially in regenerative medicine. Furthermore, the high differentiation potential of MSCs has attracted researchers to develop efficient differentiation protocols.[11,12] The therapeutic potential of these cells in treating various diseases has led researchers to focus specifically on MSCs in the field of cell therapy.[13,14]

In general, MSCs are isolated from various tissues, including bone marrow, adipose tissue, dental pulp, and Wharton's jelly. In recent years, their ability to differentiate into diverse cell lineages, such as nerve cells,[15] bone,[16] and keratocyte-like cells,[17] has been extensively investigated.

Wharton's jelly is a type of connective tissue characterized by cells surrounded by an extracellular matrix. Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) are known for their high differentiation capacity, favorable immune profile, and ease of isolation. The use of these cells raises no legal or ethical concerns.[18,19]

Regenerative medicine is an innovative approach aimed at repairing, replacing, or regenerating damaged cells, tissues, and organs. Research in this field is currently advancing at a remarkable pace, with tissue engineering and stem cell research driving significant developments over the past decade.[20]

Cannabis Sativa, commonly referred to as marijuana or hemp, is a plant native to Eastern Asia that produces a variety of chemical compounds. To date, researchers have identified 554 different compounds, including 113 phytocannabinoids and 120 terpenes, which contribute to its distinctive aroma.[21,22] Cannabidiol (CBD) is the main non-psychoactive compound derived from *Cannabis Sativa* and has been used for recreational and medicinal purposes for centuries. Unlike Δ9-tetrahydrocannabinol (Δ9-THC), CBD does not produce psychotropic effects and has demonstrated significant therapeutic potential. It influences a broad range of molecular, cellular, and organ-level processes, impacting inflammation, oxidative stress, cell viability, pain, vasodilation, and excitability, thereby modulating various physiological and pathological functions.[23] Cannabinoids, a class of chemical compounds that includes CBD, play roles in neural development and can modulate adult neurogenesis through the activation of CB1 and CB2 receptors.[24]

The establishment of a robust and efficient protocol for inducing neural differentiation remains a pivotal challenge in regenerative medicine. Current standard methods predominantly rely on expensive growth factors, which not only increase costs but also risk yielding heterogeneous cell populations due to off-target differentiation. This lack of specificity poses a significant barrier to experimental reproducibility and raises concerns for future clinical applications, where cell population purity is critical. Our study addresses this gap by investigating the potential of CBD as a novel, cost-effective, and precise inducing agent, aiming to overcome the limitations of conventional growth factor-based approaches.

The aim of this study was to investigate and analyze the efficacy of the small molecule cannabidiol in inducing the differentiation of hWJ-MSCs into neural cells. To this end, the effective dose of cannabidiol was determined using MTT and acridine orange/ethidium bromide (AO/EB) staining techniques. Cannabidiol was identified as a key factor specifically evaluated for its ability to stimulate and accelerate the differentiation of MSCs into neural-like cells. This research seeks to establish a new foundation for stem cell-based therapies, particularly in the field of neurological disorders.

Materials and Methods

Stem Cell Culture

Human Wharton's jelly mesenchymal stem cells (WJ-MSCs) were obtained from the cell bank of the Stem Cell Technology Research Center (STRC) in Tehran, Iran, and cultured according to previously established protocols.[25,26] Briefly, the cells were maintained in high-glucose DMEM (Biosera, France) supplemented with 10% fetal bovine serum (FBS) (Gibco™, USA) and 1% penicillin/streptomycin (Sigma, USA). The culture medium was changed every two days until the cells reached 75% confluence. Passage 3 of human WJ-MSCs was used in this study.

Determination of the Optimal Cannabidiol Dose

To determine the effective dose of cannabidiol (CBD) small molecule (Sigma Aldrich, Cat No: C6395), the viability of hWJ-MSCs was assessed using the MTT assay[27,28] with different concentrations of CBD (1, 5, 10, 25, 75, and 100 µg/ml). For this purpose, 5×10^3 hWJ-MSCs were seeded into each well of 96-well plates and incubated for 24 hours under controlled conditions (37 °C, 5% CO_2). Subsequently, different concentrations of CBD were added to the cells. The control group (TCPS) consisted of hWJ-MSCs cultured in basal medium (DMEM with 10% FBS and 1% penicillin/streptomycin). Cell viability was assessed on days 1, 3, and 5. At each time point, the culture medium was removed and replaced with 150 µl of MTT solution, followed by incubation for 4 hours. After removal of the MTT solution, 100 µl of DMSO (Sigma, USA) was added to the wells, and the absorbance of formazan crystals was measured using a microplate reader at a wavelength of 570–630 nm. To confirm the MTT results, acridine orange/ethidium bromide (AO/EB) staining (Sigma, USA) was performed (15, 27), followed by fluorescence microscopy imaging (Nikon, Japan).

Induction of Differentiation

To evaluate the proposed differentiation protocol, a standard differentiation medium based on previous studies was used.[29,30] This medium consisted of high-glucose DMEM supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma, USA), 2.5 µM retinoic acid (RA) (Sigma), 0.5 mM forskolin (Sigma), and decreasing concentrations of FBS (10%, 5%, and finally 2%). After 24 hours of hWJ-MSC culture, the cells were divided into two groups: a control group cultured on polystyrene tissue culture plates (TCPS group) and a group treated with 5 µg/ml cannabidiol for differentiation. For real-time PCR analysis, 1.5×10^5 hWJ-MSCs per well were used.

Evaluation of Neural Marker Gene Expression

To evaluate differentiation induction, the expression levels of neural differentiation-associated genes, *NSE*, *MAP*-2, *Oligo*-2, *β-tubulin III*, and *GFAP*, were measured at 7 and 14 days' post-induction. RNA was extracted using the RiboEX kit (Gene All Co., South Korea) according to the manufacturer's instructions. RNA quality was assessed by measuring the OD260/OD280 ratio using a NanoDrop spectrophotometer (Thermo, USA). Subsequently, cDNA synthesis was performed using a Fermentas kit. The thermal cycling protocol consisted of an initial denaturation at 95 °C for 15 minutes, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds, with a final extension step at 72 °C for 5 minutes. The expression of target genes was quantitatively analyzed relative to hypoxanthine phosphoribosyl transferase 1 (*HPRT1*), which served as an internal control. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with *HPRT1* gene.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel (2023 version) and GraphPad Prism (version 10). Data were evaluated using one-way ANOVA and t-tests, with statistical significance set at a $p \leq 0.05$. Each experiment comprised three biological replicates, and each biological replicate was performed in triplicate.

Table 1. Primers used in Real-time PCR

Gene	Primers (5′ → 3′)	Product size (bp)
NSE	F: GGAGAACAGTGAAGCCTTGG R: GGTCAAATGGGTCCTCAATG	238
MAP-2	F: AGTTCCAGCAGCGTGATG R: CATTCTCTCTTCAGCCTTCTC	97
β-tubulin III	F: GATCGGAGCCAAGTTCTG R: GTCCATCGTCCCAGGTTC	177
Oligo-2	F: GCTGTGCAAACACTTTGGGT R: AAGGGTGTTACACGGCAGAC	291
GFAP	F: GCAGACCTTCTCCAACCTG R: ACTCCTTAATGACCTCTCCATC	127
HPRT1	F: CCTGGCGTCGTGATTAGTG R: TCAGTCCTGTCCATAATTAGTCC	125

Results

Cell Viability

The cytotoxicity of cannabidiol on hWJ-MSCs and the determination of its optimal dose were evaluated using the MTT assay on days 1, 3, and 5 (Figure 1). The results clearly showed that cells exposed to 5 µg/ml cannabidiol exhibited greater viability than the control group on all evaluated days (1, 3, and 5). This increase in viability and proliferation was statistically significant on days 3 and 5. To confirm the MTT results, AO/EB staining was performed. As shown in Figure 2, cells exposed to 5 µg/ml cannabidiol demonstrated greater proliferation than the control group on days 1, 3, and 5.

Neural Differentiation

To evaluate the effectiveness of the proposed neural differentiation medium, the expression levels of key genes involved in neural differentiation induction (*NSE*, *Oligo*-2, *β-tubulin III*, *MAP*-2, and *GFAP*) were examined at 7 and 14 days. The results showed increased expression of these genes in all groups at both day 7 (Figure 3A) and day 14 (Figure 3B) compared to the control group. As shown in Figure 3A, at day 7, all neural marker genes were upregulated compared to the control group; however, the increases in *GFAP* and *MAP-2* expression were not statistically significant. The increase in *NSE* gene expression at day 7 was statistically significant compared to the control group (TCPS) ($p \leq 0.05$ and $p \leq 0.01$). On day 14 of differentiation induction, all neural marker genes showed a significant increase in expression, with the exception of *GFAP*, which also reached statistical significance ($p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.001$).

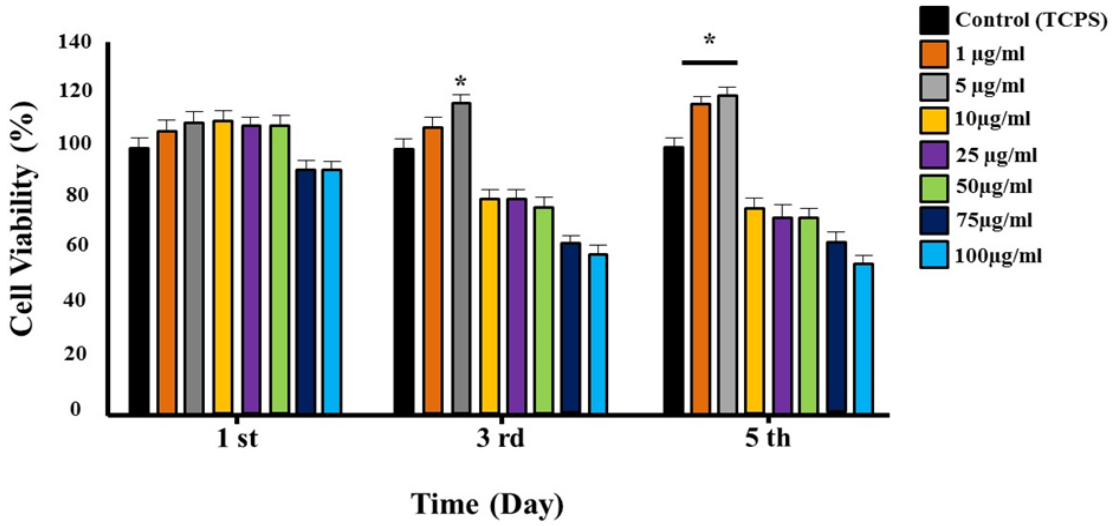

Figure 1. MTT Assay Results Showing the Effects of Various Concentrations of Cannabidiol (1, 5, 10, 25, 50, 75, and 100 µg/ml) on Treated hWJ-MSCs over 1, 3, and 5 Days in Culture Medium. All experiments were performed in triplicate ($p \leq 0.05$).

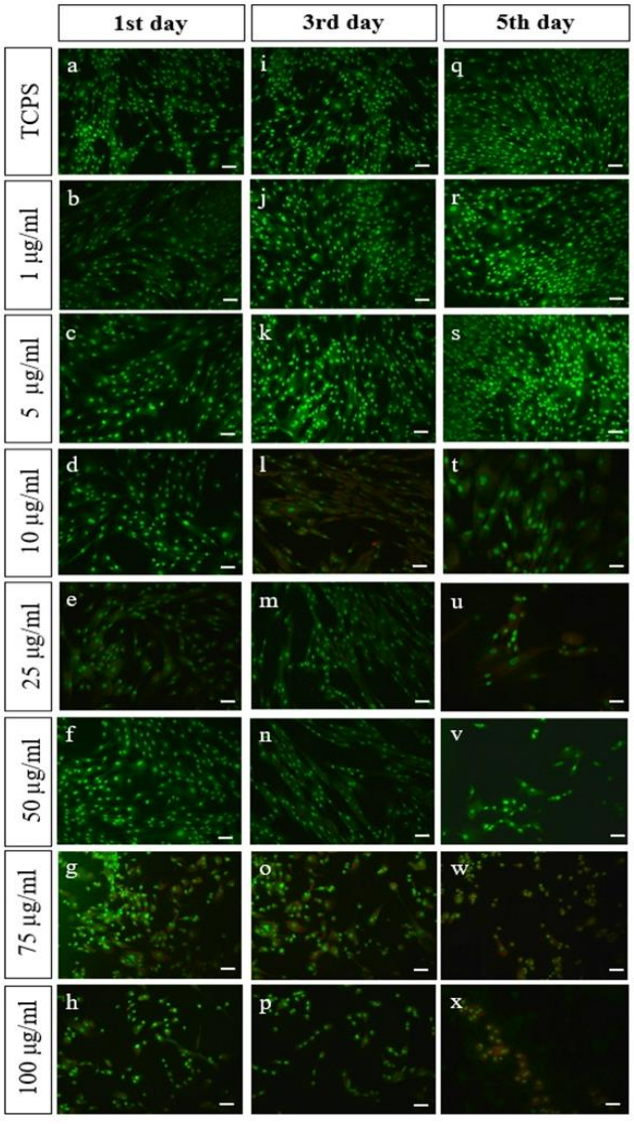

Figure 2. AO/EB Staining of Cells Exposed to Different Concentrations of Cannabidiol (1, 5, 10, 25, 75, and 100 µg/ml) for 1, 3, and 5 days. Scale bars in the images indicate 100 µm.

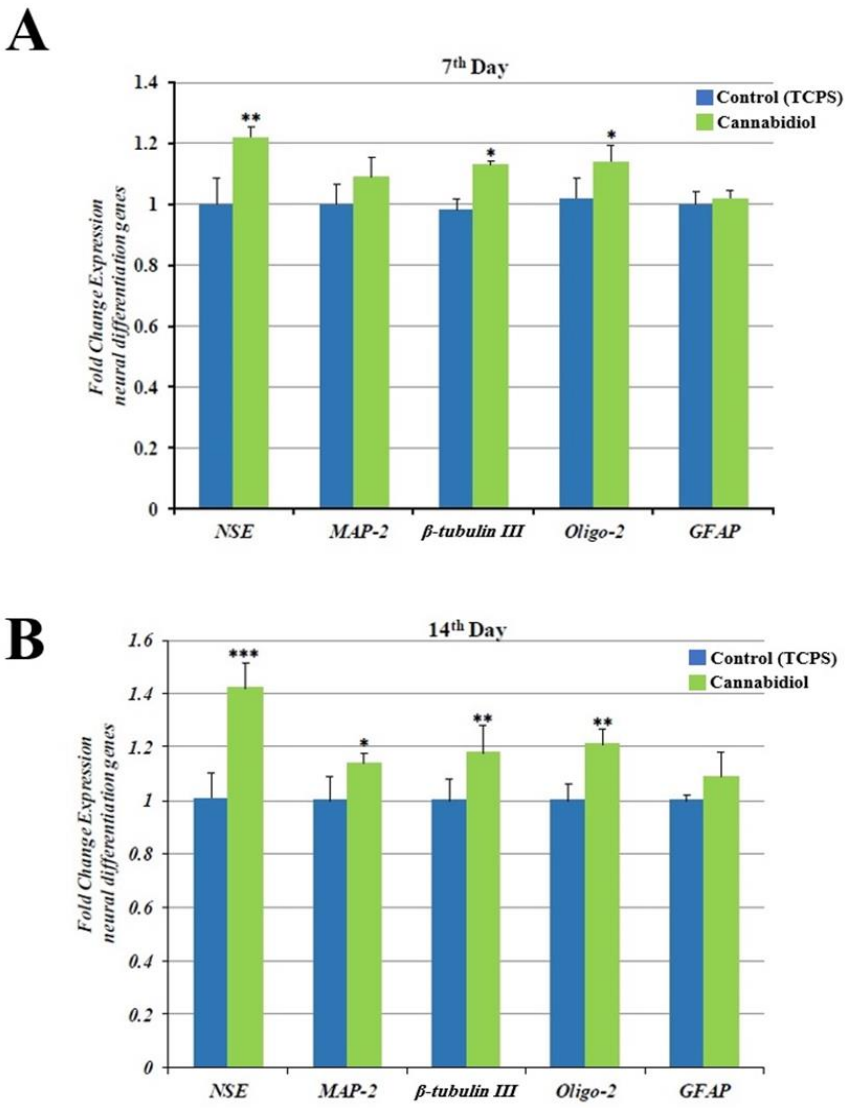

Figure 3. Relative Expression Levels of Neural Marker Genes in Differentiated hWJ-MSCs at: **A**. Day 7, and **B**. Day 14. *HPRT1* served as the reference gene. Significant differences between the two groups are indicated by asterisks, with one, two, and three asterisks representing $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

Discussion

Central nervous system (CNS) repair is a critical component of treating neurological diseases and plays a crucial role in restoring the structure and function of the nervous system. Cell therapy represents a promising strategy based on the replacement of damaged cells.[31] Over the past decade, the use of stem cells, particularly mesenchymal stem cells (MSCs), has gained attention in regenerative medicine for addressing challenges related to peripheral and central nervous system injuries.[32] MSCs originate from mesodermal tissue; these cells are multipotent and exhibit high self-renewal capacity and a unique potential to differentiate into various cell types. These properties, along with their presence in most tissues of the body, make MSCs ideal candidates for cell therapy in various diseases.[33] Qi et al. first explored the potential for cholinergic neural differentiation of bone marrow-derived MSCs as a novel approach for cell therapy and gene therapy in nerve injury.[34] Subsequently, direct transplantation of stem cells differentiated into neural cells was performed in a cerebral ischemia model,[33] accelerating research in neural regenerative medicine using stem cells.[35] Research has since expanded to include transplantation of human umbilical cord MSCs into mice with Alzheimer's disease, which significantly improved the mice's learning and memory abilities.[36] The encouraging results of these studies have strengthened researchers' belief in the applicability of this approach. However, significant challenges remain for cell therapy in nerve injury using MSCs. Poor cell survival after injection, incomplete differentiation into neural cells, and the risk of unwanted differentiation into other cell types are among the most important challenges facing this therapeutic approach.[37,38] One strategy to overcome these challenges is predifferentiation, which involves differentiating as many stem cells as possible

into neural-like cells before implantation into the tissue. Growth factors (GFs) are the primary and most important factors in the differentiation process. Nevertheless, the use of GFs is not always the optimal choice. Major challenges associated with many GFs include short half-lives, high production costs, and potential side effects.[39,40] Therefore, it seems necessary to find a cheap, suitable, and safe alternative for inducing neural differentiation.

Our objective was to develop an appropriate protocol using a differentiation cocktail containing the small molecule cannabidiol to convert WJ-MSCs into neural-like cells. A crucial aspect of this study was determining the optimal dose of cannabidiol, which was assessed using MTT assay and AO/EB staining. The results clearly indicated that a concentration of 5 µg/ml cannabidiol was not only non-cytotoxic to human WJ-MSCs but also enhanced cell viability compared to the control group (Figures 1 and 2).

In one study, CBD significantly reduced the viability of neuroblastoma cell lines in a dose- and time-dependent manner, with effective concentrations ranging from 0 to 50 µg/ml.[41] However, in the present study, concentrations of 1 and 5 µg/ml not only failed to reduce the viability and proliferation of hWJ-MSCs but also significantly increased cell proliferation on days 3 and 5 compared to the control group (TCPS). As stem cells differentiate, key genes are upregulated beyond their baseline expression levels, signaling the induction of differentiation. In this study, we evaluated the expression levels of neural marker genes to determine neural differentiation induction in stem cells. Neuron-specific enolase (NSE) is a specific marker for neuronal cells. It is a homodimeric enzyme that facilitates the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. NSE expression is particularly significant during neural differentiation, where it serves as a marker for neuronal maturation.[42]

In this study, expression of the *NSE* gene was evaluated. The results showed that *NSE* gene expression significantly increased on days 7 and 14 of differentiation induction in the CBD-treated group compared to the control group (TCPS). Recent studies conducted in 2025 have shown that when human adipose-derived MSCs (hADSCs) and hWJ-MSCs differentiate into neural-like cells, there is a significant increase in the expression of this gene and its protein.[25,43] This finding is completely consistent with and supports the present study.

Another gene that is upregulated during neuronal differentiation, along with NSE, is *β-tubulin III*, also known as *TUBB3*. The β-tubulin III protein plays a vital role in microtubule formation and organization. Expression of this gene is essential for maintaining cellular structure, particularly in neurons. Research has shown that the *TUBB3* gene is associated with neurogenesis and the development of neural structures such as axons and dendrites.[44,45] In 2023, a study by Masoumi et al. demonstrated that differentiation of hADSCs into neural-like cells is influenced by carbachol and calcium, leading to increased *TUBB3* gene expression.[11] This finding is fully supported by the results presented in Figure 3 of the current study.

In addition to the above-mentioned genes, the *Oligo2* gene plays a very important role in neural development as a neurogenic factor. Research has shown that expression of this gene promotes the differentiation of motor neurons and oligodendrocytes.[46] Previous evaluations have proven that *Oligo2* gene expression increases during the differentiation of MSCs from various origins into neural-like cells.[11,15,43] The results of the present study also confirm increased *Oligo2* expression during the differentiation process under the influence of CBD (Figure 3). These results support the effectiveness of CBD in inducing neural differentiation in human WJ-MSCs.

The *MAP-2* gene, which encodes microtubule-associated protein 2, plays a critical role in neuronal structure and function. Previous studies have shown that *MAP-2* expression is increased during the induction of neuronal differentiation.[15,29,47] In the present study, expression of this gene increased at days 7 and 14 after differentiation induction in hWJ-MSCs, and this increase was statistically significant at day 14 (Figure 3).

Glial fibrillary acidic protein (GFAP) is a vital protein encoded by the *GFAP* gene and is expressed primarily in the central nervous system (CNS). *GFAP* gene products are critical for the synthesis of part of the cytoskeleton in astrocytes.[48] In this study, as shown in Figure 3, expression of this gene increased during differentiation compared to the control group on days 7 and 14, although this increase was not statistically significant.

The results of this study suggest that CBD may be capable of inducing neural differentiation in stem cells. In a study by Soundara Rajan et al., CBD activated genes related to the proliferation and differentiation of neural progenitor cells in human gingival MSCs (hGMSCs), suggesting that CBD increases the expression of factors necessary for neural differentiation and neurogenesis.[49] The results of the present study confirm this previous report and emphasize the potential of CBD in inducing neural differentiation. Research has shown that CBD modulates CB1 and CB2 receptors by creating interactions that promote neurogenesis.[50] A 2022 study found that cannabidiol at concentrations of 5 µM and 10 µM promotes neurodifferentiation through the Akt and Erk pathways activated by CB1 signaling in the hybrid spinal cord × neuroblastoma cell line (NSC-34).[51] In the present study, a concentration of 5 µg/ml was found to be effective, demonstrating high efficacy in inducing neuro-differentiation in hWJ-MSCs *in vitro*. These results not only confirm previous findings but also highlight the significant potential of cannabidiol in promoting differentiation.

Given that CBD is more cost-effective and readily available compared to growth factors, it is proposed that, after comprehensive evaluations in *in vivo* studies, this compound be used for pre-differentiation of MSCs before transplantation into neural tissue in the field of neural regenerative medicine. Furthermore, the use of the small molecule CBD within biocompatible nanocomposite scaffolds may have a significant effect on inducing differentiation of MSCs into neural cells, potentially comparable to 3D culture methods.

Conclusion

In conclusion, this study successfully validates a specialized neural differentiation protocol for human Wharton's jelly mesenchymal stem cells (hWJ-MSCs) using the small molecule cannabidiol (CBD). Our findings demonstrate that a concentration of 5 µg/ml CBD not only preserves cell viability but also significantly enhances the expression of key neural markers at both gene and protein levels, confirming its efficacy as a non-cytotoxic and cost-effective inducer of neural differentiation. The proposed protocol offers a promising alternative to conventional growth factor-based methods, addressing challenges related to high costs, short half-lives, and potential off-target effects. By establishing CBD as an effective differentiating agent, this study provides a foundation for developing safer and more accessible strategies in neural regenerative medicine. Nevertheless, several important questions remain. Future research should focus on elucidating the precise molecular pathways through which CBD induces neural differentiation, particularly the roles of CB1/CB2 receptors and downstream signaling cascades such as Akt and Erk. Additionally, long-term *in vivo* studies are essential to evaluate the survival, integration, and functional recovery of CBD-differentiated cells following transplantation. The incorporation of CBD into biocompatible scaffolds for three-dimensional culture systems also represents a promising direction for enhancing neural differentiation and tissue regeneration. Overall, this study lays the groundwork for translating CBD-based differentiation protocols into preclinical and potentially clinical applications for neurological disorders.

Authors' Contributions

EM and AT: Conceptualization, performing laboratory procedures; RH and EM: Writing – original draft preparation, Data curation. A.S: Supervisor, and Writing – review & final editing. All authors approved the submitted version.

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Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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