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Effects of Ocrelizumab on Dental Pulp Derived Mesenchymal Stem Cells

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Abstract

Multiple sclerosis (MS) is a chronic autoimmune central nervous system (CNS) disease characterized by inflammation, demyelination and axonal damage. The most important goal in MS treatment is to provide neuroregeneration. However, current treatments only work to slow the progression of MS. Ocrelizumab is a humanized anti-CD20 monoclonal antibody used in the treatment of MS. In this study, it was aimed to investigate the effects of Ocrelizumab on the proliferation and differentiation potential of dental pulp-derived stem cells (DP-SC). For this purpose, three different (1.5 µg/mL, 15 µg/mL and 150 µg/mL) doses of Ocrelizumab were added to the normal culture medium, and then proliferation analyzes at 24, 48 and 72 hours were performed with the WST-1 assay. Ocrelizumab treatment for 24 and 48 hours was observed to dose-dependently reduce the proliferation of stem cells. At 72 hours, the efficacy of the drug on proliferation was lost at low doses. Similarly, a dose-dependent decrease in the expression of stemness marker, Rex1,was detected. The expression of the adipogenic differentiation marker ADFP, osteogenic differentiation marker RUNX2, chondrogenic differentiation marker SOX9, and neurogenic differentiation marker TUBB3 were evaluated to determine the influenceon the differentiation potential of stem cells under the drug effect. While RUNX2, SOX9 and TUBB3 expressions increased in a dose-dependent manner, there was a decrease in ADFP expression in parallel to the drug dose. According to the results of the study, ocrelizumab might have the potential to support the regenerative effects by supporting the differentiation of DP-SCs in osteogenic, chodrogenic and neurogenic.

Keywords: Multiple Sclerosis, Mesenchymal Stem Cell, Ocrelizumab, Cell Differentiation

1. Introduction

Multiple sclerosis (MS) is an autoimmune central nervous system (CNS) disease characterized by inflammation, demyelination, and axonal damage. It is a progressive disease characterized by the presence of plaques in the brain and spinal cord. MS is a chronic disease that especially affects the younger population. It is one of the most common causes of CNS diseases in young adults (1). In patients with MS, myelin sheaths, oligodendrocytes, axons and nerve cells are damaged. As a result of damage to the myelin shell, multiple plaques form in brain and spinal cord. Inflammatory plaques/lesions observed on magnetic resonance imaging (MRI) are the pathological feature of MS. According to the McDonald's diagnostic criteria, the typical lesion areas are periventricular, juxtacortical, infratentorial regions and spinal cord (2). The signs and symptoms of MS are closely related to that area, in which region of the brain and spinal cord has affected. The most common of these

symptoms are loss of sensation (paresthesias), motor spinal cord symptoms, like numbness or weakness in one or more limbs, autonomic spinal cord symptoms (bladder, bowel, and sexual dysfunction), cerebellar symptoms (dysarthria, ataxia, and tremor), optic neuritis (partial or complete loss of central vision), other eye symptoms (blurred or double vision), trigeminal neuralgia, facial myokymia, heat intolerance, fatigue, dizziness, insomnia, pain, subjective cognitive difficulties, and depression (3). The disease affects approximately 2 million people worldwide and poses a great health burden in society (4). Although the cause of MS is not known exactly, environmental conditions, like vitamin D deficiency, exposure to UVB-light, Epstein–Barr virus (EBV) infection, obesity and smoking, are associated with the occurrence of the disease. In addition, more than 150 single nucleotide polymorphisms in immune system-related genes have been identified so far that are associated with the predisposition to MS (5).

The combination of genetic characteristics and environmental conditions might increase the risk of MS. It is categorized into four subtypes based on its clinical course. There are three main clinical courses in Multiple Sclerosis: i) Clinically isolated syndrome (CIS) is defined as the first clinical attack showing inflammatory demyelination features; ii) Relapsing-remitting MS (RRMS) with varying degrees of improvement following acute relapses; iii) Secondary progressive MS (SPMS) with an initial relapsing-correcting course and characterized by an irreversible progression and accumulation of severe disability; iv) Primary progressive MS (PPMS), characterized by an uninterrupted progression from the onset of the disease. (6)

There is no curative treatment for MS, and the current treatments aim to slow the risk of relapse and the progression of disability. While these treatments provide immune modulation, they are insufficient to repair damaged tissue (7). The first DMT approved by the Food and Drug Administration (FDA) for the treatment of RRMS was interferon (IFN) beta 1b. In the following processes, other IFN agents and a new agent, glatiramer acetate (GA), were presented. In the years that followed, new therapies began to develop rapidly, including oral agents such as fingolimod, dimethyl fumarate, teriflunomide, cladribine, siponimod, ozanimod, and ponesimod, along now thoroughly used monoclonal antibodies such as natalizumab, rituximab. alemtuzumab, ocrelizumab, and ofatumumab (8). Ocrelizumab, a monoclonal antibody that selectively depletes CD20+ B cells, is the first anti-CD20 mAb approved for RMS and the first ever pharmacotherapy approved for PPMS (9).

Dental pulp stem cells (DP-SCs), which are also characterized as neural crest derived stem cells, are very similar to mesenchymal stem cells (MSCs) with respect to cell surface markers, multipotent differentiation capacity and self-renewal ability (10). Different from the MSCs, DP-SCs possess higher capacity for differentiation into neuronal cell lineage than MSCs. Therefore, they might be used as the cells for the regeneration of neurodegenerative disease of the CNS.

Ocrelizumab has been shown to have a great potential to suppress the progression of MS, but it has no effect on the healing the damage on neuronal tissue. DP-SCs might be a candidate for therapeutic cells. However, there hasn’t been any published study focusing on the interaction of Ocrelizumab with the DP-SCs. The aim of the study is to investigate the effect of Ocrelizumab, which is used in the treatment of MS, on the survival and the differentiation capacities of DP-SCs.

2. MATERIALS AND METHODS

2.1. Cell Culture

Human dental pulp stem cell (DP-SC) used in this study was obtained from the stem cell collection of the Center for Stem Cell and Gene Therapies, Research and Practice of Kocaeli University (Izmit, Kocaeli,Turkiye). Cells were cultured in DMEM (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco) and 1% Pen/Strep (Gibco) under the standard culture condition at 37 °C, 100% humid atmosphere containing 5% CO_2. When the culture reached to 70-80% confluency, cells were detached from surface by 0.25% Trypsin/EDTA solution (Gibco) at 37 °C, centrifuged at 300g for 5 min and resuspended in the culture media (1:4). The media was refreshed every two days. Ocrelizumab was diluted in sterile PBS buffer (Gibco) at pH 7.2, and final drug doses of 1.5 µg/mL, 15 µg/mL, 150 µg/mL and 1500 µg/mL were maintained in the medium.PBS buffer at pH 7.2 was used as drug vector in the assays, if not mentioned otherwise. The medium without Ocrelizumab supplementation was used as control.

2.2. Cell Viability Analysis

MTT based assay was used for the cell viability analysis. After the detachment, cells were suspended in the DMEM at 10^5 cells/mL, and 100 µL medium with cells were injected into each well of 96 well-plates. Following the overnight culture at 37 °C, the culture medium was removed, and cells were incubated in the basal DMEM medium supplemented with 10% Wst-1 solution (Roche, Mannheim, Germany) under the standard culture condition for 30 minutes. Finally, absorbance was recorded at 450 nm using spectrophotometer (VersaMax, Molecular Devices,CA, USA).

2.3. Gene Expression Analysis

Following the cell culture and the drug treatment, total RNA was isolated by the RNA isolation kit (GeneJet RNA Isolation Kit, Thermo Scientific), according to the manufacturer's directions. The single strand cDNA was synthetized by Transcriptor High Fidelity cDNA Synthesis Kit (Roche) with respect to the provided protocol. The gene expression analysis was carried out in the Real Time PCR (LightCycler480-II) using the DNA SYBR Green I Master (Roche) with gene-specific primers. Data were analyzed with the LC480 SW1.5 software (Roche). ActB gene amplification was used as housekeeping gene in the calculations.

2.4. Neurogenic Differentiation

The previously defined method was used to differentiate DP-SCs into neuronal cells (11). Following the culture, cells were seeded on the laminin-coated culture plate (3,000/cm^2). The culture medium was replaced with neurogenic differentiation medium (DMEM supplemented with 5% FBS, 1% Pen/Strep, 1X N2 medium (Gibco), 1X B27 medium (Gibco), 1mM retinoic acid (Sigma)). The differentiation medium was refreshed every two days. After 8 days of incubation, the cells were examined for differentiation by immune staining against anti-Tubb3 (Tubulin beta 3) antibody.

2.5. Immunofluorescence Staining

After the neurogenic differentiation, cells were fixed with 4% paraformaldehyde for 15 minutes, and washed with PBS (Thermo). Then, samples were blocked by 1.5% block serum (Santa Cruz Biotechnology, Heidelberg, Germany). Primary antibody against Tubb3 was diluted according to the manufacturer's recommendation and incubated at 4°C for 16 hours. Anti-Tubb3 antibody was used as the marker for neuronal cell differentiation. For immunofluorescence studies, it was incubated with TexasRed-conjugated secondary antibody for 1 hour at room temperature and covered with mounting

medium (UltraCruz Mounting Medium with DAPI, Santa Cruz Biotechnology). Stained cells will be examined under a fluorescent microscope (Leica DMI 4000 Microsystems).

2.6. Statistics Analysis

Collected data were analyzed using SPSS 10.0 statistical program. All data were expressed as means ± standard deviation. Statistical analysis of the data was determined by one-way ANOVA, followed by Turkey tests. P-value (P) < 0.05 denoted to statistically significant difference.

3. Results:

In the control cell culture medium without Ocrelizumab or vector, the DP-SCs have the cell morphology of thin and spindle-like structure similar to mesenchymal cells (Figure 1).The addition of the drug into the medium had no visible effect on thecell morphologyat the doses of 1.5 and 15 µg/mL. At higher doses, the cells became elongated in shape compared to the control or vector, but there was a slight alteration.No cell death was visiblein any of the cell cultures.After 48 hours,it was observed that DP-SCs did not lose their typical fibroblast-like morphology (Figure 1).

According to the results of WST-1, the cell proliferation was suppressed in parallel with the dose increase in the first 24 hours ($p<0.05$). The suppression of cell proliferation by Ocrelizumab lost its effect after 48 hours, and it disappeared after 72 hours(Figure 2). The observed decrease in the cell proliferation after 72 hours was at the same level of the vector group, possibly due to the effect of pH on the cell culture. After 48 hours, the cell viability was improved to 119.9% ±5.5 ($p<0.01$) compared to control.

The expression of the stemness marker, REX1, is related with the self-renewal and the differentiation in adult stem cells. The REX1 expression significantly increased at 1.5 doses at 24 and 48 hours, compared to control. On the other hand, the expression of REX1 decreased at higher drug doses. The difference in decrease was most significant at 150 µg/mL ($p<0.01$ and Figure 3). This difference was about 1.7-fold after 24 and 48 hours, but the reduction in the gene expression was as low as 2.6-fold after 72 hours compared to the control. The stemness factor's expression was significantly improved in PBS at pH 7.2 after 24 hours by 2.2-fold, and suddenly decrease to the control levels after 48 hours.

The Ocrelizumab effect on the differentiation potential of DP-SCs was evaluated only for the osteogenic, adipogenic, chondrogenic and neurogenic cell lines. The differentiation markers were analyzed by Real Time PCR after 24, 48 and 72 hours of Ocrelizumab treatment.

The expression of the early osteogenic differentiation marker, RUNX2, was downregulated under the effect of the Ocrelizumab at 1.5 µg/mL ($p<0.001$ and Figure 4) after 24,48 and 72 hours. At higher doses, the downregulation could not be observed, except for the cell cultures for 72 hours. The RUNX2 expression was downregulated about 2-fold ($p<0.005$) respect to control, regardless of the drug doses.

The expression of SOX9, a chondrogenic differentiation marker, did not altered significantly after 24 hours on the contrary to the RUNX2 expression. However, the SOX9 expression was suppressed at 1.5 µg/mLOcrelizumab doseafter 48 hours, and suddenly increased significantly after 72 hours ($p<0.005$). The most remarkable change in SOX9 expression was observed for 150 µg/mL Ocrelizumab dose after 24 hours and 48 hours by 4.1- and 3.2-fold increase, respectively (Figure 4-B). Even after 72 hours, the SOX9 expression level was as high as about 2.4-fold with respect to control. Although the drug application generally improved the osteogenic and chondrogenic differentiation of DP-SC, Ocrelizumab

showed negative effect on the adipogenic differentiation capacity of DP-SCs. The expression of ADFP, a marker for theadipogenic differentiation, was downregulated in the presence of the drug, regardless of the drug dose compared to control or vector group (Figure 4-C).

The expression of TUBB3, a neurogenic differentiation marker, increased significantly in parallel with the increasing Ocrelizumab dose ($p<0.005$ and Figure 4-D). Although the vector group showed an improved TUBB3 expression (1.3-fold) with respect to control, the experimental groups with varying Ocrelizumab doses showed about 2.3-fold higher TUBB3 expression. Furthermore, Ocrelizumab was added into the neurogenic differentiation of DP-SCs as supplement. The 1.5 and 15µg/mL doses of Ocrelizumab was shown to improve the protein level significantly. Although the 150 µg/mL drug dose had highest impact on the TUBB3 expression, the cell viability was adversely affected, and cells could not be cultured for the defined period of neurogenic differentiation for 8 days. Following the neurogenic induction, the cells were stained by immunofluorescence methodagainst the anti-TUBB3 antibody (Figure 5). The TUBB3 could not be detected in the control (Figure 5-C0) without drug. The drug supplementation at low dose ofOcrelizumab (1.5µg/mL, Figure 5-C1) in the normal culture group significantly improved the TUBB3 expression in the cells without any significant loss of cell viability. At 15 µg/mL dose in the normal culture group (Figure 5-C2), there was still intensely stained cells, but the number of cells was decreased in parallel to the loss of cell viability. After 8 days of neurogenic differentiation, the differentiated group without drug (Figure 5-D0) showed the TUBB3 protein expression, but without any significant morphological change. The TUBB3 expression was still high in the drug supplemented differentiation groups (Figure 5-D1,D2), but the morphological alteration was visible. They showed neuron-like morphology after differentiation and Ocrelizumab treatment enhanced this effect.

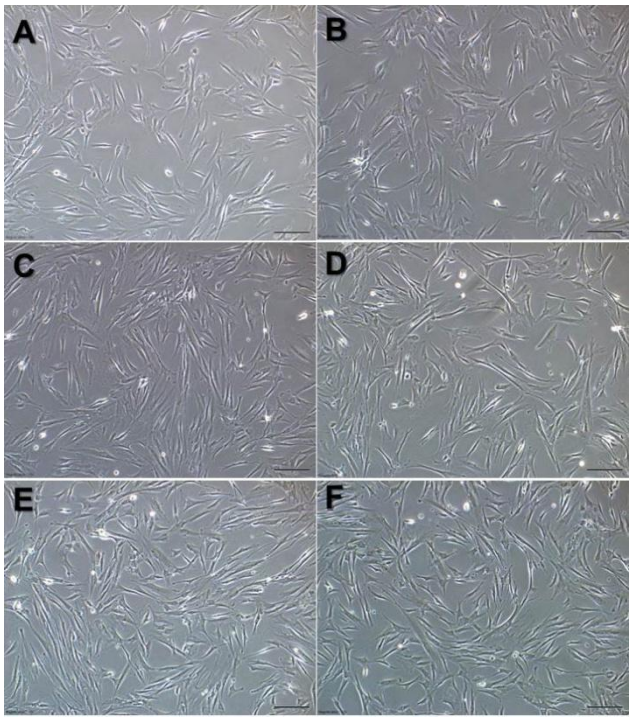
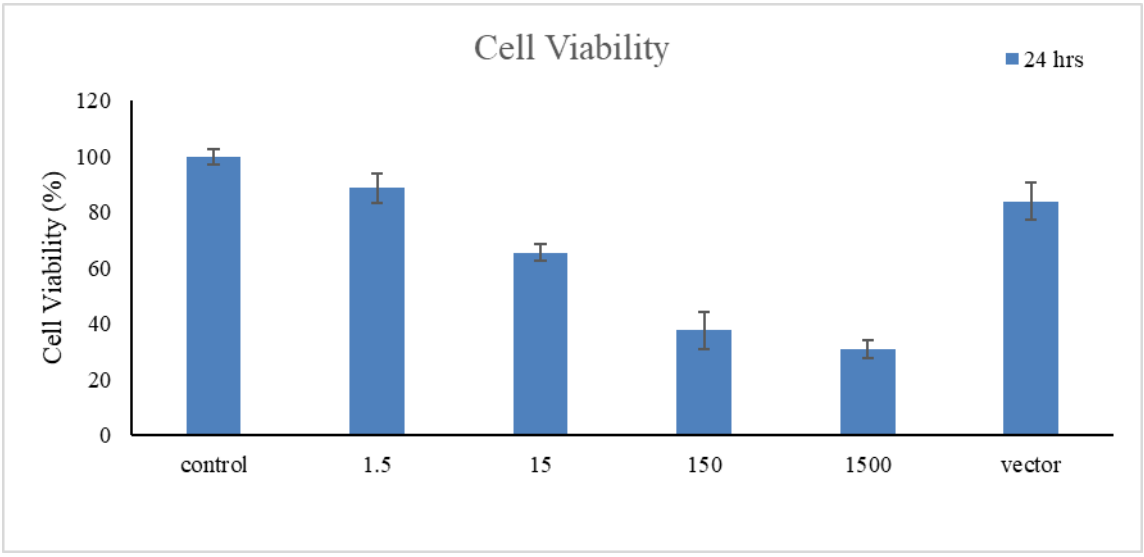
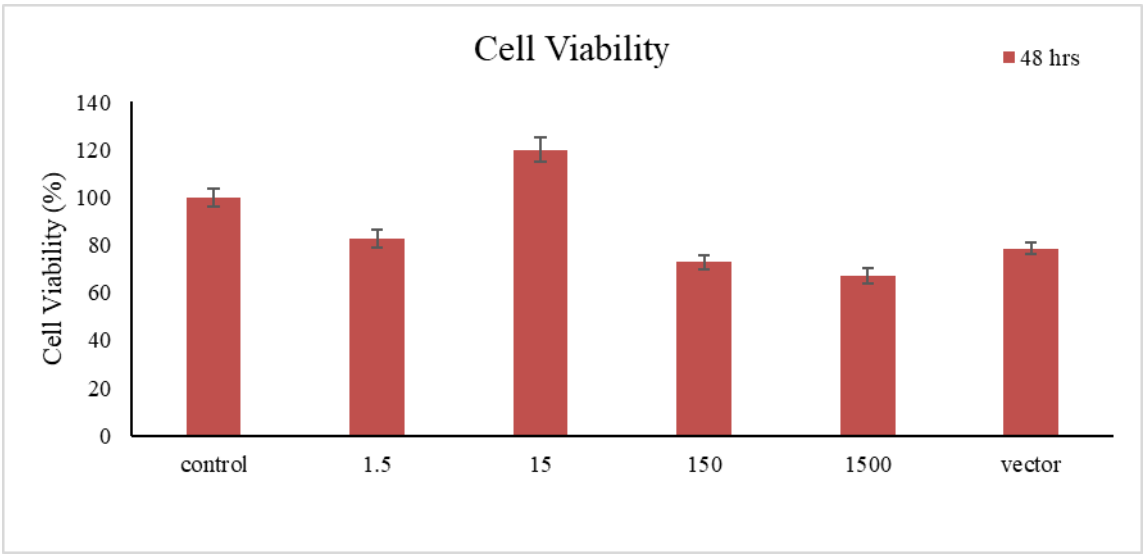


Figure 1: Cell morfology of human dental pulp stem cells (DP-SC) in the medium supplemented with different doses of Ocrelizumab, after 48 hours of culture. **A**, 1.5 µg/mL; **B**, 15 µg/mL; **C**, 150 µg/mL; **D**, 1500 µg/mL; **E**, control; **F**, vector (PBS). Scale bar: 100 µm.





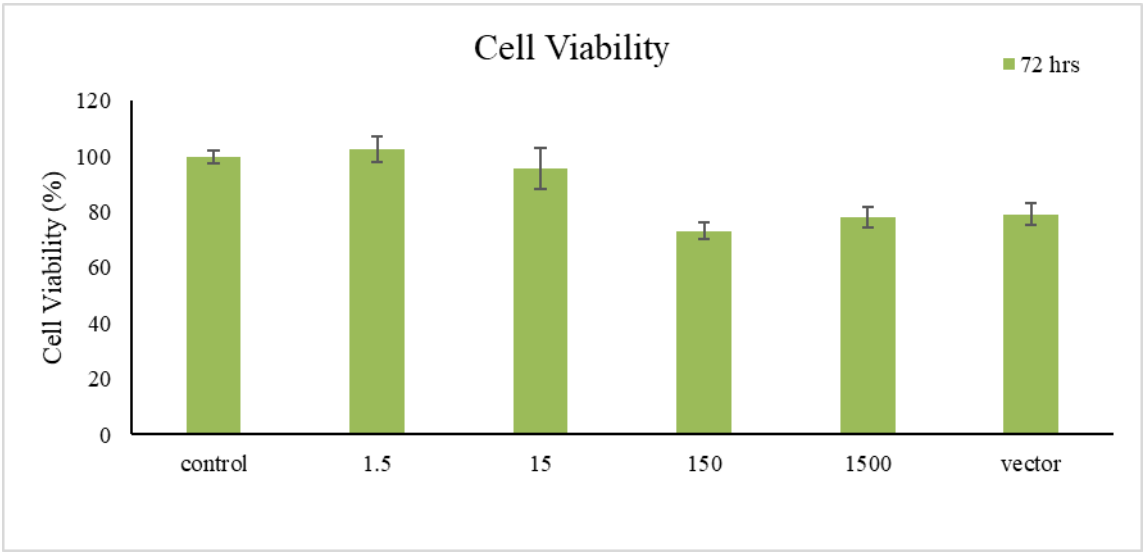


Figure 2: Cell viability index. The effects of Ocrelizumab were examined with Wst1 in a dose-dependent manner.(1.5 – 1500 µg/mL) at 24, 48 and 72 hours. The untreated cell group was taken as a control.

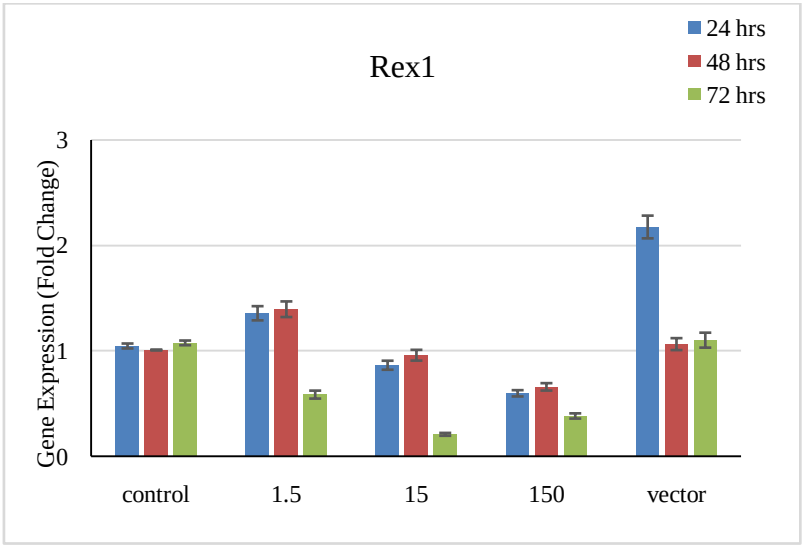


Figure 3: The effect of Ocrelizumab at 1.5, 15, and 150 µg/mL doses on REX1 (stemness factor) expression was determined by Real Time PCR at 24, 48, and 72 hours.

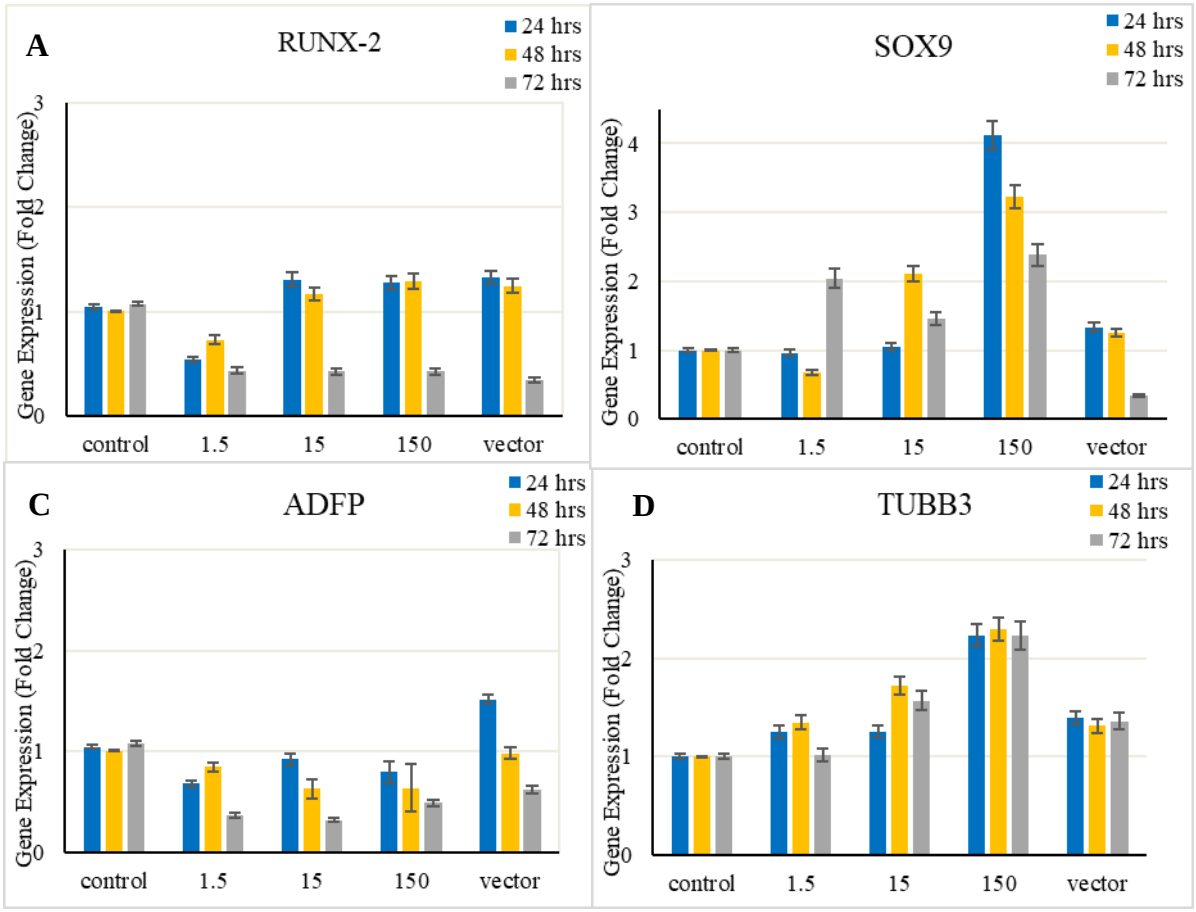


Figure 4: After 24, 48, and 72 hours of Ocrelizumab at 1.5, 15, and 150 µg/mL doses, expression of Osteogenic (RUNX2), Chondrogenic (SOX9), Adipogenic (ADFP) and Neurogenic (TUBB3) markers in undifferentiated DP-SCs was determined by real-time PCR.

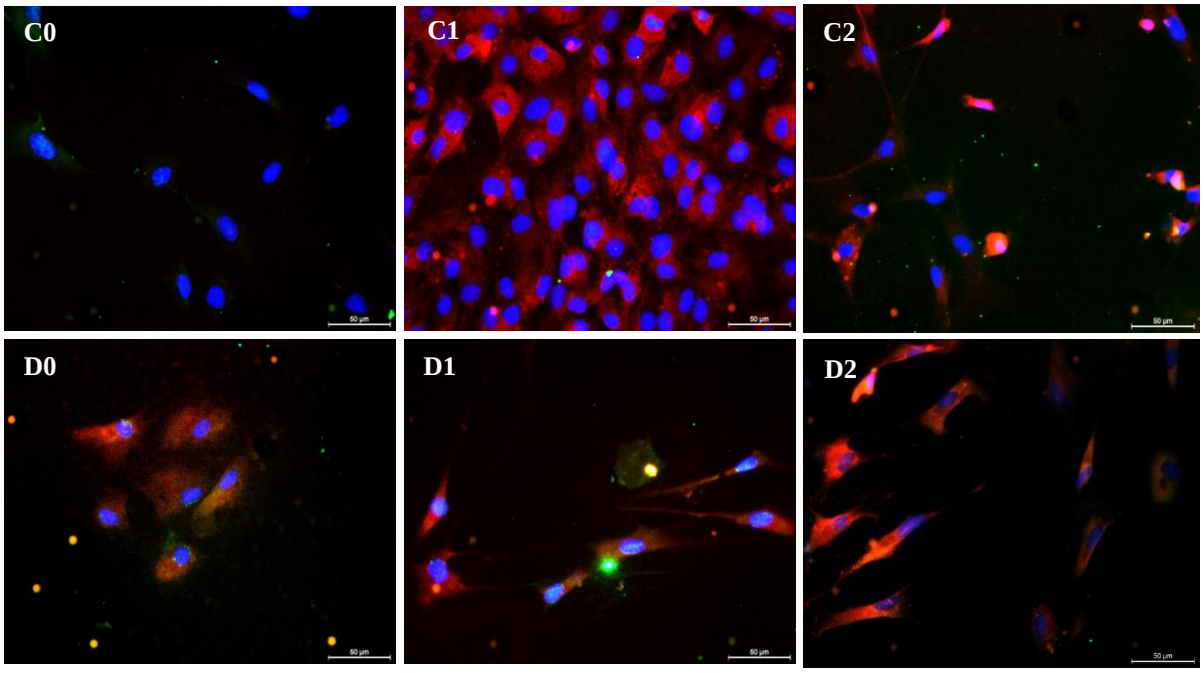


Figure 5: Immunofluorescent staining of DP-SCs againstanti-TUBB3 antibody (red). **C0:** Ocrelizumab free in normal culture condition, **C1:** 1,5 µg/mL Ocrelizumab in normal culture condition, **C2:** 15 µg/mL Ocrelizumab in normal culture condition, **D0:** After neurogenic differentiation without Ocrelizumab, **D1:** After neurogenic differentiation with 1,5 µg/mL Ocrelizumab and **D2:** After neurogenic differentiation with 15 µg/mL Ocrelizumab. Nuclei were stained by DAPI (blue). Scale bars, 50 µm.

4. Discussion:

Ocrelizumab is an anti-CD20 monoclonal antibody that was proposed to be used in the treatment of MS and gives encouraging results in the selective depletion of CD20expressing B cells (9). Stem cell therapy is an effective treatment in regenerative medicine and a promising new approach in cases where current treatments are insufficient. Treatment with stem cells can be effective by many mechanisms, including neuroprotection, immunomodulation, and neuroregeneration. In a clinical study performed on 16 patients, placental-derived mesenchymal stem cells were used and disease progression was reported to be halted (12). In another clinical trial conducted on 48 patients, autologous bone marrow mesenchymal stem cells were administered intrathecally and intravenously, and it was reported that the improvement in patients might be due to their neuroprotective, neuroregenerative and immunomodulatory effects (13).Human dental pulp stem cells were shown to have high angiogenic and neurogenic potential to differentiate (14,15). In those papers, DP-SCs were reported to express the early neurogenic differentiation markers, like tublin-beta. These characteristics supported cells to differentiate more effectively into neuron-like cells. Although DP-SCs are very similar to mesenchymal stem cells, they differ from them by their expression of neuronal specific markers (10). Interestingly, the Ocrelizumab positively affect the neuronal differentiation as it was for the osteogenic and chondrogenic differentiations.In DP-SCs, it was shown that ERK/MAPK is necessary for neuronal differentiation (16). In another study, the MAPK and TGF-β signaling pathways were shown to play a central role in the neurogenic differentiation, and the difference in those pathways between the DP-SCs and MSCs make DP-SCs more potential to differentiate neuron-like cells (17).The enhanced MAPK and TGF-β signaling in DP-SCs might be the reason for being the target of Ocrelizumab. Although the function of CD20 was not clearly known in cells, it

was shown to be related to TGF-β signaling in other cells (18). It is still unclear the exact mechanism, but the Ocrelizumab might have to potential to improve the neuronal differentiation.

In our study, neural crest-derived dental pulp stem cells, which have higher neurogenic differentiation capacity than other sources, were preferred and it was shown that ocrelizumab, which is used in the treatment of MS, increases the regenerative effects of stem cells.While the ease of obtaining stem cells and their unique mechanisms have led to the success of clinical studies with them, there are some limitations to working with stem cells.Standard culture conditions, treatment dosage, and number of cells to be used have not been determined for their clinical use, properly.In this sense, our study brings an innovative perspective to the literature in terms of revealing the possible effects of ocrelizumab on stem cells.

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