Aksaray Üniversitesi Tıp Bilimleri Dergisi
ASUJMS, 4(2) : 01-05

Araştırma Makalesi

Serotoninin Adipoz Doku Kökenli Mezenkimal Kök Hücrelerin Proliferasyonuna Etkisinin Wnt-β Sinyal Yolağı Üzerinden İncelenmesi

Investigation of the Effect of Serotonin on Proliferation of Adipose Tissue-Derived Mesenchymal Stem Cells via Wnt-β Signaling Pathway

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ÖZET

Amaç: Hücresel tedaviler açısından mezenkimal kök hücreler (MKH'ler), sahip oldukları özellikler nedeniyle en çok çalışılan hücrelerdir. Özellikle klinik çalışmalarda MKH'lerin devamlılığı önemlidir. Wnt sinyali, MKH'lerin çoğalması ve farklılaşmasının düzenlenmesinde hayati bir rol oynar.

Materyal ve Metot: Bu çalışmada adipoz doku kökenli MKH'lerin (AD-MKH'lerin) proliferatif özelliği üzerine Serotoninin (5-hydroxytryptophan [5-HT]) etkisinin Wnt-β sinyal yolağı üzerinden araştırılması amaçlanmıştır. Çalışmada AD-MKH'ler kullanıldı. İlk olarak, Serotoninin 1-100 µM arasında değişen konsantrasyonlarının AD-MKH'lere etkisi MTT testi ile analiz edildi. Hücreler üzerine apoptotik etkisi Annexin V/PI ve analizi ile belirlendi. WNT2, AXIN ve CTNNB1 genlerinin ekspresyon düzeyleri moleküler düzeyde qPCR ile analiz edildi.

Bulgular: MTT analizi sonucu 48. saatte 75 µM serotoninin proliferasyonu azalttığı gözlendi. 48 saatlik 75 µM serotonin uygulaması sonrası apoptotik hücre oranı %2.05'e çıkarken, erken apoptoz evresindeki hücre oranı %0.77 olarak tespit edildi. WNT2, AXIN ve CTNNB1 gen ekspresyonlarının kontrol grubu ile karşılaştırıldığında, WNT2 ve AXIN up-regüle olurken ($p<0.001$) CTNNB1 ekspresyonunun ise serotonin uygulanmış hücrelerde down-regüle olduğu belirlendi.

Sonuç: Sonuçlar serotoninin mezenkimal kök hücrelerin proliferatif etkisini Wnt sinyal yolağı üzerinden artırabildiğini göstermektedir. Ancak in vivo ve ileri moleküler düzeyde çalışmalarla desteklenmesi gerekmektedir.

Anahtar Kelimeler: Mezenkimal kök hücre, serotonin, Wnt-β sinyal yolağı.

ABSTRACT

Aim: Mesenchymal stem cells (MSCs) are the most studied cells due to their properties in terms of cellular therapies. In particular, the continuity of MSCs is essential in clinical studies. The Wnt signal plays a vital role in regulating the proliferation and differentiation of MSCs. In the present study, the effect of serotonin (5-hydroxytryptophan [5-HT]) on the proliferative properties of human adipose tissue-derived MSCs (hAT-MSCs) was investigated through the Wnt-β signaling pathway.

Materials and Methods: First, the effect of serotonin concentrations ranging from 1-100 µM on AD-MSCs were analyzed by MTT test. Annexin V/PI analysis was performed to determine the apoptotic effect on the cells. Finally, the expression levels of WNT2, AXIN, and CTNNB1 genes were analyzed at the molecular level by qPCR.

Results: The MTT analysis revealed that 75 µM serotonin reduced proliferation at 48 hours. After 48 hours of treatment with 75 µM serotonin, the apoptotic cell rate increased to 2.05%, while the early apoptotic cell rate was determined as 0.77%. Compared with the control group, WNT2 and AXIN gene expressions were up-regulated ($p<0.001$), while the CTNNB1 expression was down-regulated in the cells treated with serotonin.

Conclusion: The results show that serotonin can increase the proliferative effect of mesenchymal stem cells via the Wnt signaling pathway. However, further in vivo and advanced molecular studies are required to support these findings.

Keywords: Mesenchymal stem cell, serotonin, Wnt -β signaling pathway.

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Geliş Tarihi : 17.05.2023
Kabul Tarihi : 07.08.2023

INTRODUCTION

Mesenchymal Stem Cells (MSCs) are self-renewing and multipotent cells that can differentiate into various mesenchymal cell types such as chondrocytes, osteoblasts, adipocytes, myocytes, and neurons (1). Various molecular factors, including bone morphogenetic proteins, Wnt proteins, and severe transcription factors, are responsible for the sustainability and differentiation of MSCs (2). MSCs have been extensively studied for the past 30 years due to their exciting cell biology, broad clinical potential, and rapidly growing "central building block" in tissue engineering. In addition, MSCs have unique properties such as easy growth in a culture dish, internal differentiation potential not found in other cells, and abundant production of beneficial growth factors and cytokines (3).

Serotonin, or 5-hydroxytryptamine (5-HT), is a neurotransmitter and a hormone. Serotonin is made from an essential amino acid called tryptophan via the enzymes tryptophan hydroxylase and dopa decarboxylase (4). Serotonin acts on its receptors or small GTPases in target cells to exert its effects (5). Serotonin's physiological and pathological importance is well known in the central nervous, gastrointestinal, and cardiovascular systems. Additionally, studies have shown that serotonin is mitogenic in various standard and malignant cells, including fibroblasts, osteoblasts, and vascular endothelial cells (6, 7).

One study on the effects of serotonin on mesenchymal stem cells reported that fluoxetine, which is used as an antidepressant and a serotonin reuptake inhibitor (SSRI), affected the differentiation of mesenchymal stem cells to adipocytes and osteoblasts, and that each class of antidepressants had a varying effect on MSC differentiation (8). However, the number of studies on the proliferation or differentiation of mesenchymal stem cells for clinical use is limited. For this reason, we aimed to explore the effect of serotonin, which is known to have positive effects, on the proliferation of human adipose tissue-derived mesenchymal stem cells via the Wnt-β catenin signaling pathway. For this purpose, mesenchymal stem cells of adipose tissue origin were cultured. MTT analyzed their proliferation status, apoptosis status was analyzed by Annexin V\PI method, and Wnt-β catenin signaling pathway genes' expression was analyzed by qPCR after serotonin application.

MATERIALS AND METHODS

Cell culture

The study used hAT-MSCs (Sigma, SCC038), previously purchased commercially for another research and stored frozen. For the experiments, cells were cultured at 37°C and 5% CO^2 with a medium composed of DMEM (Gibco, 11966025), 10% fetal bovine serum FBS, (Gibco, 16000044), 0.2% Primocin (Invivogen, ant-pm-1) and 1% Glutamax (Gibco, 35050061). Cells were controlled daily and sub-cultured when the culture flask reached 70% confluency (approximately 2-3 days).

Cytotoxicity analysis

Serotonin was dissolved in 0.1% DMSO at concentrations ranging from 1 µM to 100 µM. Cells were seeded in a 96-well culture plate at $5x10^3$ cells per well in 200 µL of the medium. After cell attachment, 100 µL of the medium was removed and replaced with 100 µL of serotonin at the indicated concentrations. Cells were then incubated for 24 and 48 hours. At the end of the incubation periods, MTT solution was added to each well and further set for 3-4 hours. After removing the medium, 100 µL of DMSO was added, and the plate was kept in the dark for 15 minutes and measured at 540 nm absorbance using a microplate reader. Cell viability was calculated relative to the control group.

Annexin V/PI analysis

Cells were counted and seeded onto 6-well plates with $3x10^5$ cells per well in 2 ml of growth medium. After 24 hours of incubation, 1 ml of the medium was removed and replaced with 75 µM serotonin in 1 ml of fresh medium. Following incubation, cells were washed twice with 1x PBS and centrifuged for 5 minutes. After centrifugation, cells were resuspended in 100 µL of binding solution and pipetted. Next, cells were incubated in the dark with 5 µL of AnnexinV and 5µL of Propidium Iodide (PI). Subsequently, 400 µL of the binding solution was added and analyzed using a flow cytometer.

qPCR analysis

Total RNA extraction was performed from stem cells treated with serotonin (GeneAll® Hybrid-RTM, Seoul, Korea). The obtained RNAs were converted to complementary DNA (cDNA) using the cDNA synthesis kit (Atlas Biotechnology, Ankara, Turkey). For real-time PCR reaction, SYBR Green-containing master mix solution (RT² SYBR Green qPCR Mastermix, Qiagen, Hilden, Germany) and primers specific to WNT2, AXIN, CTNNB1, and GAPDH genes (Table 1) were used to analyze gene expression on Rotorgene Q5 plex+HRM Real-Time PCR device (Qiagen, Hilden, Germany). The evaluation was examined by the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer sequences used for qPCR analysis.

Gene Name	Primer sequence (Forward; Reverse)
GAPDH	CACCCTGTTGCTGTAGCCATATTC GACATCAAGAAGGTGGTGAAGCAG
WNT2	TTTGGCAGGGTCCTACTCC
AXIN	CCTGGTGATGGCAAATACAA
CTNNB1	ATGGAGCTCTCCGAGACAGA

Statistical Analysis

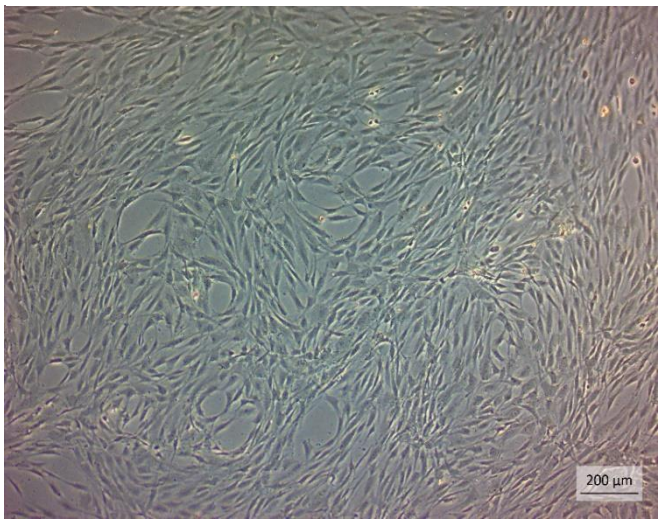
The obtained data were evaluated using one-way ANOVA with post-hoc Tukey HSD. The significance threshold values of $p<0.05$, $p<0.01$, and $p<0.001$ were used for differences between the study and control groups. All statistical analyses were performed using GraphPad Prism 7.0 software.

RESULTS

Cell Culture

During the daily examinations of cells with phase-contrast microscopy, it was observed that the cells had star-shaped or spindle fibroblast-like morphology (Figure 1).The cells were monitored daily under a microscope until the required number for the study was reached, and every three days, the culture medium was changed. When they reached 70-80% confluence, they were removed with trypsin-EDTA and passaged by dividing them into new culture dishes at a ratio of 1/3.

Figure 1. Phase-contrast microscope images of adipose tissue-derived mesenchymal stem cells adhered to the culture dish on the 3rd day after being placed into culture. The mesenchymal stem cells of adipose tissue origin were observed to have a spindle or star-shaped fibroblast-like morphology (Scale bar=200 µm).



MTT cytotoxicity analysis

Cellular cytotoxic effects of serotonin concentrations were determined at 24 and 48 hours using MTT analysis. No significant changes were observed in cell viability in the group treated with 1µM serotonin compared to the control group. However, partial reduction in cell viability was observed at 48 hours in groups treated with 75µM serotonin (Figure 2).

Annexin/PI analysis

It was observed that 1.66% of the control group cells were apoptotic, and 0.98% were in the early apoptotic stage at 48 hours. After 75 µM serotonin application for 48 hours, the rate of apoptotic cells increased to 2.05%, while the rate of cells in the early apoptotic stage was determined as 0.77%. With the application of 100 µM serotonin at 48 hours, the rate of apoptotic cells increased to 3.35%, and the rate of cells in the early apoptotic stage increased to 1.43% (Figure 3).

Figure 2. MTT analysis. % Viability values of serotonin concentrations calculated by the

MTT method at the 24th and 48th hours (ns means non-significant).and result.

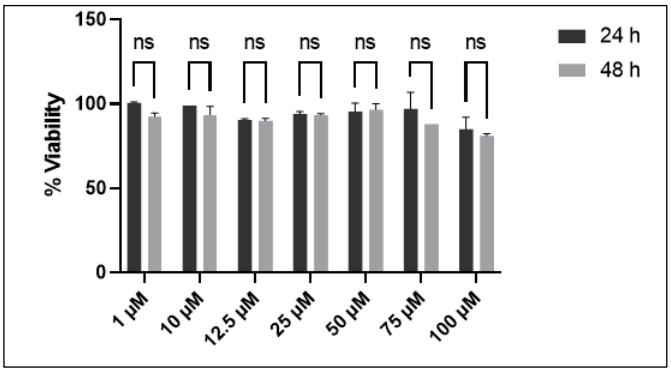
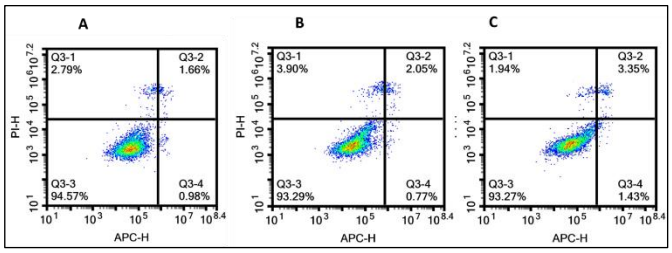


Figure 3. Annexin V/PI results of the Serotonin-treated cells. A: Untreated cells, B: 75 µM of Serotonin-treated cells, C: 100 µM of Serotonin-treated cells (Q1=Necrosis, Q2=Late apoptosis, Q3=Viability, Q4=Early apoptosis).



Results of gene expression analysis

In qPCR analysis, while WNT2 and AXIN gene expressions were up-regulated ($p<0.001$) compared to the control group, it was determined that the expression of CTNNB1 was down-regulated in cells treated with serotonin (Figure 4) (Table 2).

Figure 4. Expression profile of WNT2, AXIN, and CTNNB1 genes in mesenchymal stem cells of adipose tissue origin. Compared to the control group, it was determined that WNT2 and AXIN were up-regulated, while the expression of CTNNB1 was down-regulated (n = 3, mean ± SD, *** $p<0.001$) (Experiment was repeated three times) (ns means non-significant).

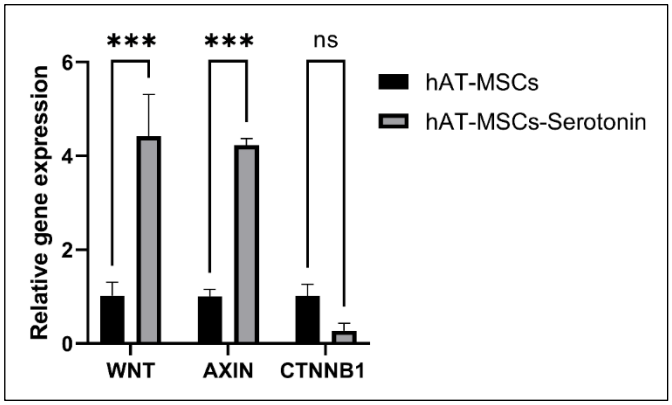


Table 2. Relative gene expression results.

Relative gene expression/cell	hAT-MSCs		hAT-MSCs-Serotonin	
	mean	sd	mean	sd
WNT	1,02	0,28	4,42***	0,89
AXIN	1,00	0,14	4,22***	0,14
CTNNB1	1,01	0,24	0,27	0,15

DISCUSSION

Subsequent studies have shown that MSCs exist in alternative tissue sources, have an adhesive, fibroblast-like population, and can differentiate into different cell types (9,10). Due to their properties and ease of isolation, they are the most preferred cell source for gene therapy and tissue engineering applications. In addition, the number of clinical studies is increasing day by day. However, the regulatory mechanisms of self-renewing and differentiation of MSCs at the molecular level, overcoming replicative senescence, and inducing desired differentiation are still significant problems awaiting solutions in the clinic (11). Therefore, our study, conducted with adipose tissue-derived MSCs that have a crucial place in cell physiology and clinical studies, will conduce to the literature in this area.

Various studies have shown the importance of Wnt signaling in the fate decisions of MSCs. Additionally, the effects of Wnt signaling are contextual (12). The functional variation of Wnt proteins depends on the specific surface receptor they interact with and the subsequent cellular signaling pathway that is activated (13). However, it is still unclear which cell types produce Wnt proteins. β-catenin is the main component of the canonical Wnt signaling pathway; in the absence of Wnt, β-catenin associates with APC (adenomatous polyposis coli) and Axin, facilitating its phosphorylation by casein kinase Iα (CKIα) and glycogen synthase kinase 3β (GSK3β), and subsequent degradation by ubiquitin/proteasome via β-TrCP (14). Therefore, we investigated the expression levels of Wnt signaling pathway genes that play an essential role in the proliferation and differentiation decisions of MSCs.

Due to the positive results in studies using hematopoietic stem cells, we conducted research on our hypothesis that serotonin, which has never been studied before, may increase the proliferation of adipose tissue-derived hematopoietic stem cells. Serotonin has been suggested to mediate the bidirectional interactions between the nervous and immune systems. It has significant activities on inflammation and immunity by affecting the actions of almost all mature blood cell types. Serotonin is an immune signal between dendritic cells and T cells and increases the ex vivo expansion of CD34+ hematopoietic stem and progenitor cells. Platelets release 5-HT in response to signals, including damaged endothelium or ischemia (16). Since most (> 90%) of the serotonin in the blood is stored in the dense granules of platelets, serotonin in the hematopoietic system is closely related to platelets and megakaryocytes. It is known that serotonin stimulates megakaryopoiesis through 5-HT2 receptors (16, 17). Most studies on serotonin are associated with Serotonin reuptake inhibitors (SSRI), the most common class of drugs for depression. The pharmacological mechanism of these drugs is related to blocking the 5-HT (serotonin) transporter, which leads to the inhibition of 5-HT reuptake and increases synaptic 5-HT levels (18). However, there are limited studies on the effect of serotonin on mesenchymal stem cell proliferation. In the present study, there is no significant decrease in the viability of AT-MSCs after applying various serotonin concentrations. After using 75 μM and 100 μM serotonin doses that partially decreased viability, the expressions of WNT2 and AXIN genes, which activate the Wnt signaling pathway, were up-regulated ($p<0.001$). In contrast, the expression of CTNNB1 was down-regulated. We believe the reason for the increased expression of WNT2 and AXIN genes is that the Wnt signaling pathway associated with proliferation induced by serotonin in AT-MSCs may be practical. The decrease in CTNNB1 expression may be due to the activation of the non-canonical pathway of this pathway.

CONCLUSION

Conclusions

The evaluation of the Wnt signal by the cell can vary depending on the microenvironment in which the stem cell is located and the influence of other signals provided by the microenvironment. Therefore, future studies should address the microenvironment of MSCs along with serotonin and the involvement of different signaling pathways.

Conflict of interest statement

The authors declared no conflict of interest

Funding

The authors claimed that this study received no financial support.

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