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Melatonin as a Potential Therapeutic Tool in Allergic Rhinitis Induced by House-Dust Mite

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ABSTRACT

Melatonin is known as an important regulator of circadian rhythm in humans. In the literature, there are no studies evaluating the efficacy of melatonin in the management of allergic rhinitis (AR) or nasal polyps (Np). Np tissue was taken from nasal cavity and mucosal tissue (Mu) was taken from the nasal septal area. Melatonin (25-200nM) and Mite Allergen (2.5-12.5%) were prepared in complete media. Cell viability, apoptosis, intracellular reactive oxygen species production and gene expression levels were determined. Our results showed that there is no toxic effect of Melatonin, Mite and their combination which was given to Np-MSCs and Mu-MSCs. Melatonin significantly reduced reactive oxygen species levels in both mite-treated Np-MSCs and Mu-MSCs. Indoleamine 2,3-dioxygenase level was significantly decreased in melatonin-treated cells. Cyclooxygenase-1 level was significantly decreased in melatonin-treated healthy and allergic Np-MSCs while there was no significant difference in 100 and 150nM Melatonin-treated Mu-MSCs. Interestingly, 50nM Melatonin significantly increased Cyclooxygenase-1 level in Mu-MSCs. 50, 100 and 150nm Melatonin significantly decreased Interleukin-6 level in Mite-treated Np-MSCs. In addition, 100 and 150nM Melatonin significantly decreased Interleukin-6 level in Mite-treated Mu-MSCs. Melatonin has well-established anti-oxidant and anti-neoplastic activity, could be a promising therapeutic agent in the treatment of AR and nasal polyposis.

Keywords: Melatonin mite allergen, Allergic rhinitis, Nasal polyp tissue, Mucosal tissue, Mesenchymal stem cell.

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Introduction

Allergic rhinitis (AR), an inflammatory disease affecting nasal mucosa tissues, is seen in 10–30% of the population, with the highest prevalence in young adults and children [1]. Nasal obstruction is one of the most typical symptoms of AR, often has a circadian rhythm in terms of severity; the worst is at night and the early morning. Other typical symptoms of AR such as runny nose and sneezing, are more severe in the early morning; possibly in conjuncture with the high levels of inflammatory cells and mediators during this time period [2]. The reason for the circadian rhythm in AR is not yet fully understood.

It is also known that atopic diseases, such as asthma, nasal polyposis (NP) and AR generally coexist in the same patient [3]. Also, the patients with NP are often affected by the same problems, and considering that nasal polyps develop due to the presence of chronic inflammation, it is quite possible that their pathophysiology is influenced by similar mechanisms. This has been suggested to be associated with factors such as oxidative stress, genetic predisposition and environmental stimuli that trigger the underlying inflammatory reactions [4]. In addition, the measurement of Cyclooxygenase 1 (*COX1*), indoleamine 2,3-dioxygenase (*IDO*) and Interleukin-6 (*IL-6*) amounts, which are detected in high amounts in AR, can be used as an indicator in AR and asthma models [5,6]. One of the major problems described in clinical guidelines for AR is sleep disturbance and patients often have a dysfunctional circadian rhythm that affects cortisol and melatonin secretion [7].

Melatonin (N-acetyl-5 methoxideriptamine) is an indolamine which is synthesized mainly in the pineal gland [8], but extrapineal melatonin synthesis has been described in many other sites of the body including the brain, retina, harderian gland, ciliary body, lens, thymus, airway epithelium, bone marrow, immune cells, gonads, placenta, gastrointestinal tract

and skin [9]. Melatonin is known to be an important regulator of circadian rhythm in humans, and is a direct radical scavenger in addition to its indirect antioxidant effects on the cell membrane. In both in vivo and in vitro studies, melatonin has been shown to be a potent endogenous free radical scavenger that acts as an anti-inflammatory agent [9]. Melatonin stimulates several antioxidative enzymes such as superoxide dismutase, glutathione peroxidase and glutathione reductase, and thereby protecting cell membranes from lipid peroxidation by neutralizing toxic radicals [10]. In addition, previous study has shown that melatonin has neuro-immunological effects and may affects immunomodulatory activity in allergic diseases [11]. However, the potential use of melatonin in atopic diseases is rarely considered. On the other hand, melatonin has also been reported to play an important role in the pathogenesis of AR [12]. Melatonin and its precursor, 1-tryptophan, have been shown to decrease serum total IgE and IL-4 levels [13]; therefore, chronic inflammatory activity seen in patients with AR and NP may be compensated by the effects of melatonin. This effect can be explained as, by increasing the amount of melatonin, the suppression of indoleamine 2,3-dioxygenase (IDO), a tryptophan dehydrogenase enzyme, may increase the amount of melatonin precursor 1-tryptophan [6]. Melatonin causes partial inhibition of nuclear factor-kappa B (NF-κB) expression which is a trigger of pro-inflammatory activity and down-regulation of inducible nitric oxide synthase (iNOS) activity in lung tissue in an experimental model of asthma [14]. Plasma melatonin levels are reportedly reduced in patients with AR [15], atopic dermatitis (AD) [3] and in the exacerbation period of patients with bronchial asthma (BA).

There are no studies evaluating the efficacy of melatonin in the management of AR or nasal polyps, which have no definitive treatment. In the present study, we aimed to investigate the effect of melatonin in the treatment of Nasal-polyp Mesenchymal Stem Cells (Np-MSCs) and Mucosal Mesenchymal Stem Cells (Mu-MSCs).

Materials and Methods

Tissue Collection and Ethical Consideration

Nasal polyp tissue was taken from the nasal cavity in functional endoscopic sinus surgery of allergic rhinitis patient and mucosal tissue was taken from the nasal septal area of the non-allergic patient during septorinoplasty surgery while reconstructing the nasal cavity. Written consent was obtained from 2 patients; nasal polyp with allergic rhinitis was 35 years old, white man, had grade 3 nasal polyps in both nasal cavities originated from osteomeatal complex, had house dust mite allergy, non-allergic patient was 38 years old, white woman, had minimal septal deviation and had negative prick test. We did prick test (intradermal allergy test) for both patients. Institutional Ethical approval was obtained from Istanbul Yeniyüzyıl University Medical Faculty Ethical Committee (Ethic no :09.11.2018/032). All phases of the work were carried out in accordance with the Helsinki Declaration and Good Clinical Practice Guide.

Isolation, Cell Culture Conditions and Characterization

Cells were cultured in complete media that consists of Dulbecco's modified essential medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS, Gibco, Carlsbad, CA) and 1 % of penicillin, streptomycin, and amphotericin (PSA, Gibco, Paisley, UK) and incubated at 37°C and 5 % CO2 in a humidified incubator. When cells had enough confluency, they were trypsinized and incubated with primary antibodies diluted in phosphate-buffered saline (PBS, Gibco, Paisley, UK) for 1 h. Conjugated antibodies against CD14, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105 and CD117 (Abcam, Bristol, UK) were used to determine their mesenchymal stem cell surface profile according to criteria of International Mesenchymal Stem Cell Committee. Cells were washed with PBS. The flow cytometry analysis of cells was performed using a Becton Dickinson FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometry system.

Cell Viability Assay

Cell viability was measured by the 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) assay (CellTiter96 Aqueous One Solution; Promega, Southampton, UK) according to the manufacturer's instructions. Five different concentrations of melatonin between 25 and 200 nM (25, 50 100, 150 and 200nM) and Mite Allergen (Allutard, 10000U) between 2.5 and 12.5 % (2.5, 5, 7.5, 10 and 12.5 %) from 10.000U stock solutions were prepared in complete media. Np-MSCs and Mu-MSCs (passage number 2-4) were seeded onto 96-well plates (Corning Plasticware, Corning, NY) at a concentration of 5,000 cells/well and cells were treated with melatonin and mite allergen alone and in combination for 24, 48 and 72 hours. MTS solution was prepared according to the manufacturer's instructions and the cells were incubated for 2 hours in the dark. Cell viability was measured by ELISA Plate Reader (Biotech, USA) at 490nm absorbance.

Annexin-V and Propidium Iodide Staining

Determining the apoptotic effect of melatonin and mite allergen alone and in combination on Np-MSCs and Mu-MSCs, cells were stained with fluorescent annexin V (annexin V- FITC) and propidium iodide (PI) using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions. The cells were seeded onto 6-well-plates (2x105 cells/well) and incubated for 24 hours. After the incubation period, cells were treated with melatonin and mite allergen alone and in combination. After 24 hours, cells were harvested and resuspended in Annexin V binding buffer. Then, Annexin V-FITC and Propidium Iodide (PI) staining solution were added to each experimental group and incubated at RT for 20 minutes in the dark. Samples were analyzed using BD FACS Calibur Cell Sorting System (BD Biosciences Pharmingen; San Diego, CA, USA).

Determination of Intracellular Reactive Oxygen Species (ROS) Production

In order to measure the ROS levels on Np-MSCs and Mu-MSCs and investigate the effects of melatonin and mite allergen alone and in combination dichlorodihydrofluorescein diacetate (DCFH-DA) (Abcam, Cambridge, UK) was used as an intracellular ROS probe according to manufacturer's instructions. Cells were seeded onto black 96 well plates with a clear bottom (Greiner F-bottom chimney 96-well plate) at 10,000 cells/well. Then, the cells were treated with melatonin and mite allergen alone and in combination at different concentrations for 16 hours. After washing with PBS, cells stained with DCF-DA and CellTracker™ Red CMTPX Dye in serum free media and incubated at 37ºC for 30 min in the dark. Fluorescence intensities (Ex/Em; 495/529 nm, 577/602 nm) were measured with Varioskan LUX fluorescence microplate reader (Thermo Fisher Scientific, NY, USA).

Reverse Transcription and Quantitative Polymerase Chain Reaction (qPCR)

Total RNAs were isolated from melatonin and mite allergen alone and in combination treated groups by using High Pure RNA-isolation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. cDNA was synthesized from isolated Total RNAs by using High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany). RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, NY, USA) staining method was used to determine mRNA levels of the COX1, IDO, IL-6 and 18S RNA genes. cDNAs were mixed with primers and SYBR Green Master Mix. The primer sequences for qPCR were: for COX1 5'-GAGTTTGTCAATGCCACCT-3'(forward) and 5'-CAACTGCTTCTTCCCTTTG-3'(reverse); IDO 5'-CGCTGTTGGAAATAGCTTC-3'(forward) and 5'-CAGGACGTCAAAGCACTGAA-3'(reverse); IL-6 5'-GACAACTTTGGCATTGTGG-3'(forward) and 5'-ATGCAGGGATGATGTTCTG-3'(reverse); 18SRNA 5'-CGGCTACCACATCCAAGGAA-3' (forward) and 5'-GCTGGAATTACCGCGGCT-3'(reverse). The 18S RNA housekeeping gene was used for normalization of data. All RT-PCR experiments were performed using iCycler RT-PCR system (Bio-Rad, Hercules, CA). Additionally, PCR conditions were performed as initial denaturation at 95 for 3min, 39 cycle of following steps; denaturation at 95 for 30sec, annealing at 58 for 45 sec, extension at 72 for 30 sec and lastly final extension at 72 for 3 min.

Statistical Analysis

The data were statistically analyzed using one-way analysis of variance (ANOVA) with Tukey post-hoc test. The values of $p<0.05$ were considered statistically significant.

Results

Isolation and Characterization of Mesenchymal Stem Cell-Derived from Nasal Polyp and Mucosal Tissues

Np-MSCs and Mu-MSCs, which were successfully isolated and expanded from nasal polyp and mucosal tissue samples (Figure 1a, d), showed fibroblast-like cell morphology (Figure 1b, e). Isolated and cultured cells at passage 3 to 6 were characterized for their MSC surface markers using flow cytometry. Nasal polyp and Mucosal tissue derivative cells were characterized for the surface markers including CD29, CD14, CD31, CD44, CD34, CD45, CD73, CD90, CD105, and CD117 by flow cytometry. Cells were positive for CD29, CD44, CD73, CD90, and CD105, MSC surface markers, whereas they were negative for CD14, CD34, CD45, and CD117 hematopoietic stem cell (HSC), surface markers, and for CD31, endothelial cell marker (Figure 1c, f).

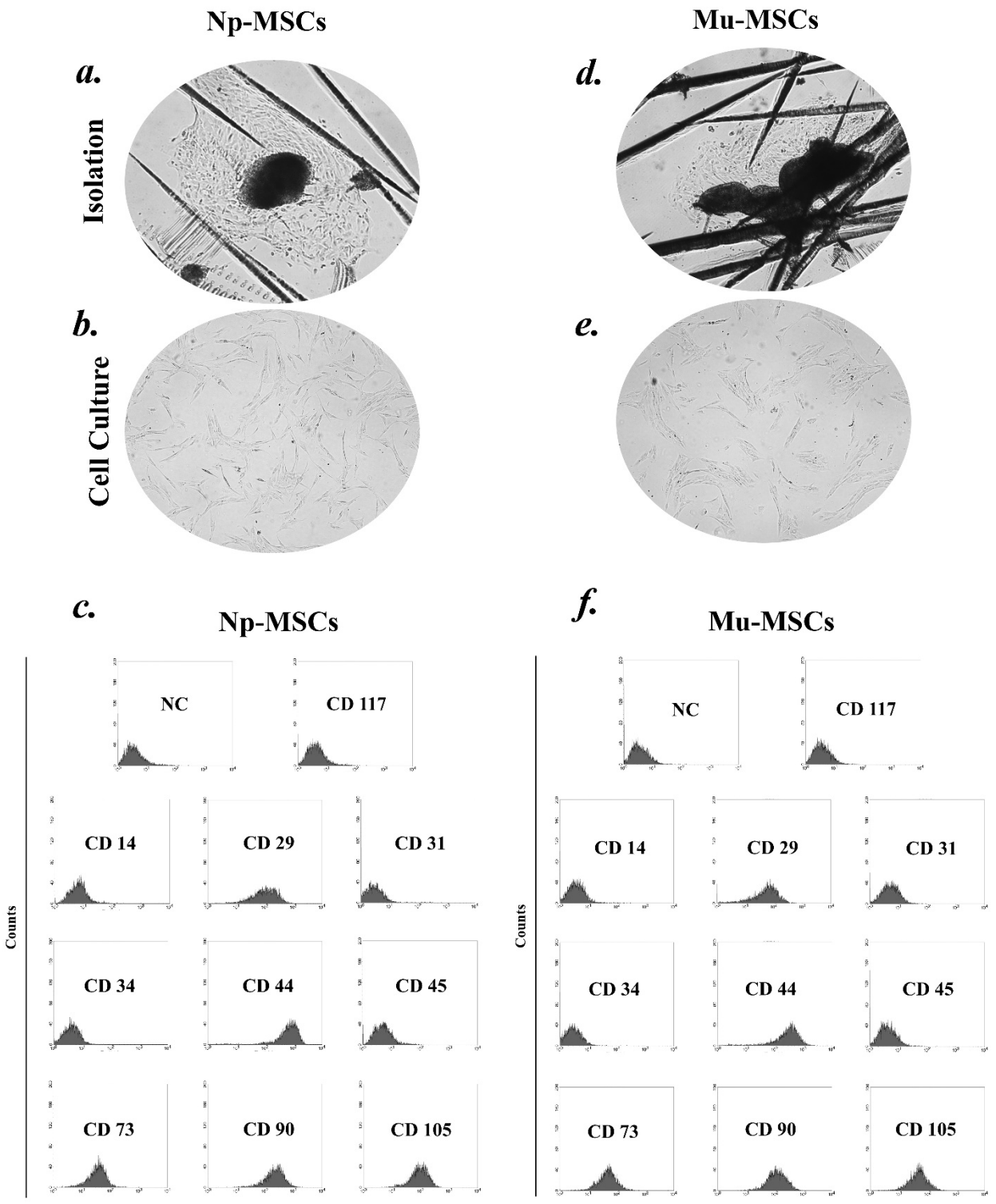

Figure 1. Cell images from isolation and culture process of the Np-MSCs (a, b and c) and Mu- MSCs cells (d, e and f). Characterization of MSCs Nasal polyp and Mucosal tissues.

Cytotoxicity of Melatonin and Mite Allergen W/O Combination

The cytotoxic effect of different Melatonin (25, 50, 100, 150 and 200 nM) and Mite Allergen (25, 50, 75, 100, 125 µg/mL) concentrations on Np-MSCs and Mu-MSCs viability were tested for 2 days at 24 and 48-h time points using the MTS assay. The results showed that there is no toxic effect of Melatonin (Figure 2a and d), Mite Allergen (Figure 2b and e) and their combination (Figure 2c and f), which was given to Np-MSCs and Mu-MSCs cells at different doses. On the contrary at some doses, they were increased the survival of cells significantly when compared to only growth media treated group.

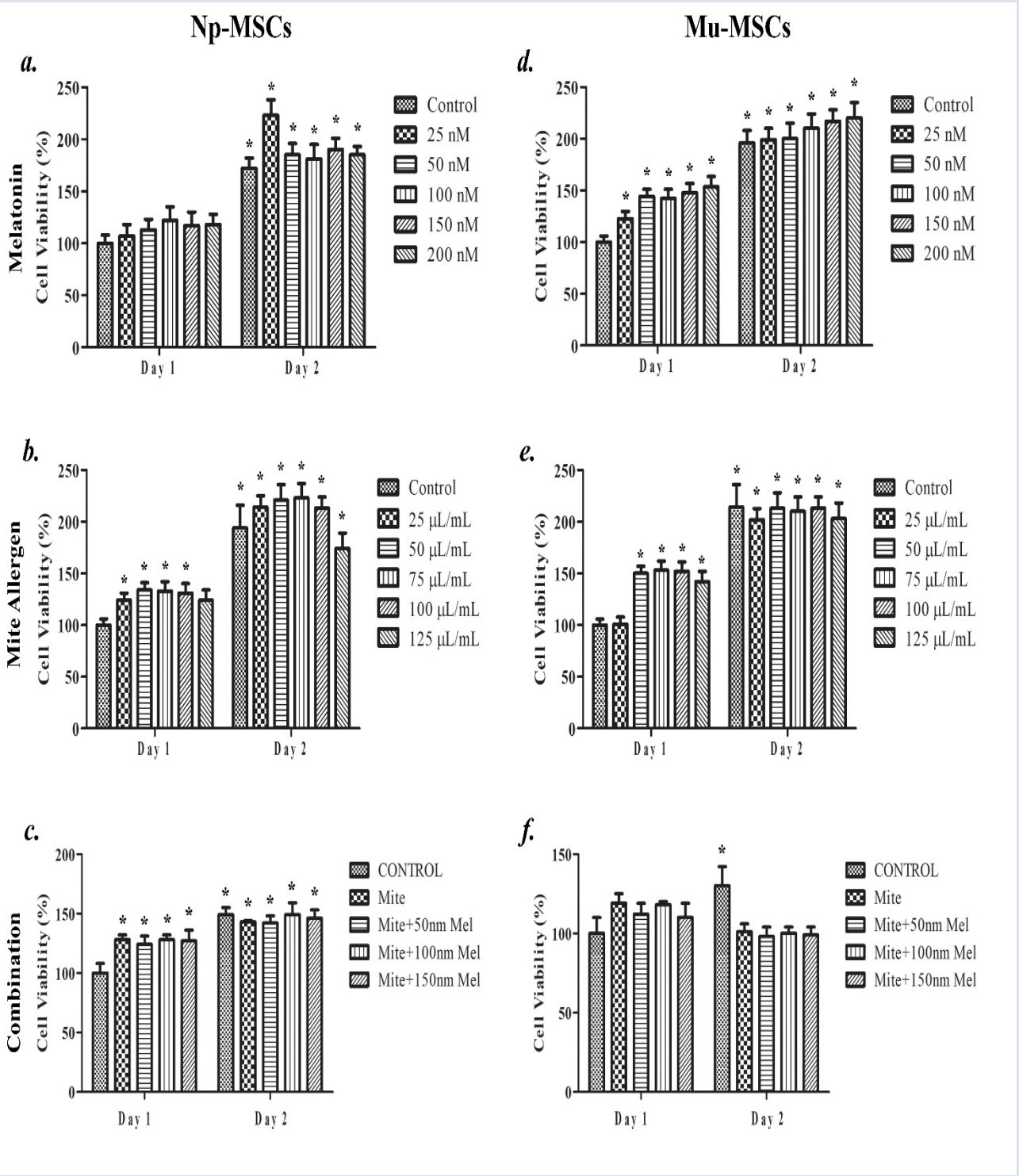

Figure 2. Cell images from isolation and culture process of the Np-MSCs (a, b and c) and Mu- MSCs cells (d, e and f). Characterization of MSCs Nasal polyp and Mucosal tissues.

Evaluation of Reactive Oxygen Species Levels in Melatonin-Treated Allergic Cells

In order to determine the indirect inflammation response, the ROS levels of NP-MSCs and Mu-MSCs were measured by the treatment of melatonin and mite allergen alone and in combination. Intracellular ROS levels were detected by using DCFH-DA fluorescence dye. Three different dose of melatonin (50, 100 and 150 nM) treatment of the allergic cell models reduced the ROS levels significantly in both mite allergen treated Np-MSCs and Mu-MSCs for all doses (Figure 3) compared to mite allergen treatment alone.

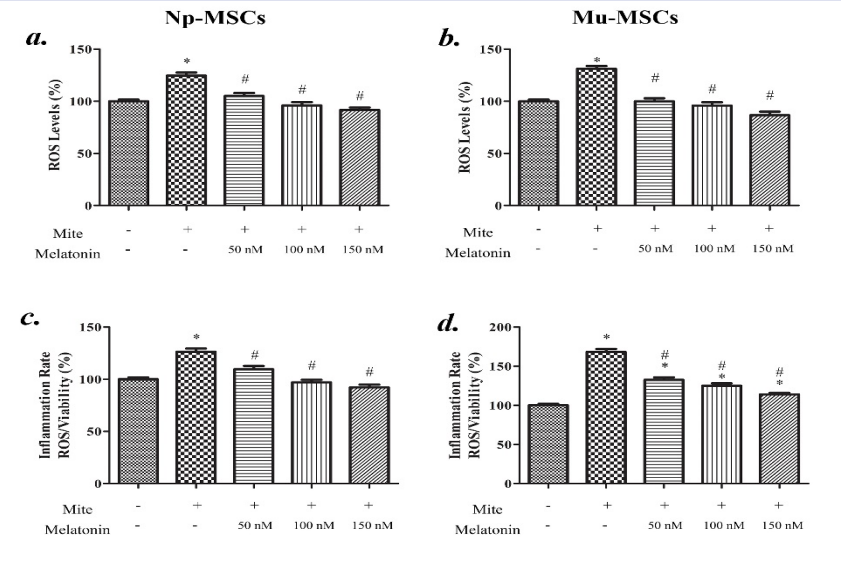

Figure 3. Cell images from isolation and culture process of the Np-MSCs (a, b and c) and Mu- MSCs cells (d, e and f). Characterization of MSCs Nasal polyp and Mucosal tissues.

Melatonin Treatment can Reduce Inflammation and Allergic Response to Allergic Cells in a Gene Level Manner

To determine the suppressive effects of Melatonin treatment on the allergic cell models, inflammation and allergic response related *IDO*, *COX1* and *IL-6* mRNA levels were evaluated by relative RT-PCR. Levels of *IDO* mRNA was decreased significantly in all 3-melatonin treated allergic cells (Figure 4a, d) when compared to Mite allergen treated Np-MSCs and Mu-MSCs. *IDO* level was significantly decreased when Np-MSCs were treated with 150nM Melatonin and Mu-MSCs were treated with 100 and 150 nM compared to only growth media treated group (Figure 4a, d). *COX1* gene expression were significantly decreased in melatonin treated healthy and allergic Np-MSCs groups (Figure 4b) while there was no significant difference in 100 and 150 nM Melatonin treated Mu-MSCs cells (Figure 4e). Interestingly, 50 nM Melatonin significantly increased *COX1* level in Mu-MSCs (Figure 4e). 50, 100 and 150 nm Melatonin treatment significantly decreased *IL-6* gene level in Mite-exposed Np-MSCs (Figure 4c). In addition, 100 and 150 nM Melatonin significantly decreased *IL-6* gene level in Mite-exposed Mu-MSCs compared to 50 nM Melatonin (Figure 4f).

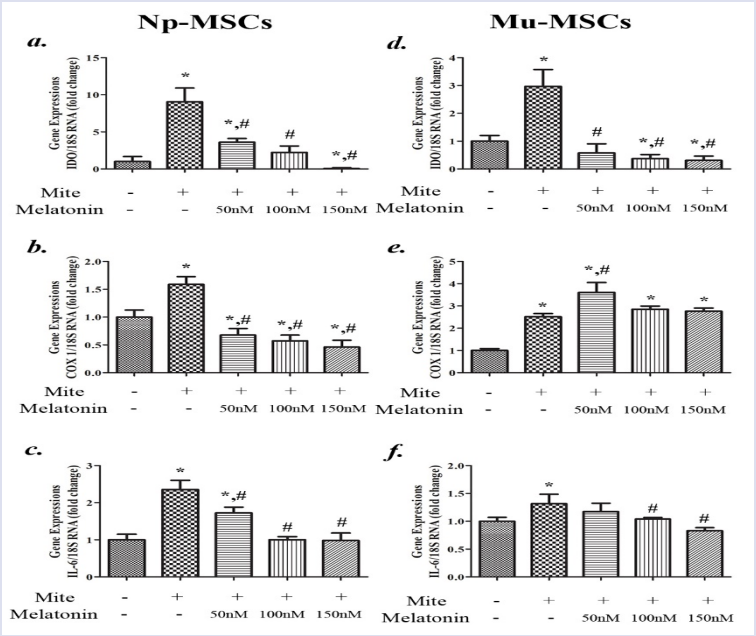

Figure 4. Cell images from isolation and culture process of the Np-MSCs (a, b and c) and Mu- MSCs cells (d, e and f). Characterization of MSCs Nasal polyp and Mucosal tissues.

Discussion

Atopic rhinitis is one of the most common chronic inflammatory diseases in all age groups (16). The pathophysiology of AR is based on IgE-mediated hypersensitivity reactions and the release of Th2-mediated cytokines, similar to other atopic diseases. However, since there is no permanent curative treatment, research has remained active on this topic, especially in terms of the elucidation of pathophysiology and treatment options. In the current literature, there are promising findings with new substances which directly affect development of disease or reduce AR severity (17,18). In our study, the effectiveness of melatonin in AR treatment was evaluated (as well as its possible cytotoxicity) by *IL-6*, *COX-1*, *IDO* and ROS levels. Our findings revealed that melatonin significantly reduced *COX-1* gene expression in Np-MSCs groups and also ROS intensity, *IDO* and *IL-6* gene levels in both Np-MSCs and Mu-MSCs groups –without any toxic effects. Thus, our results show that melatonin has significant anti-inflammatory and anti-oxidant activity in the presence of chronic inflammatory conditions of the respiratory tract, including AR and nasal polyps.

For nearly four decades, several studies have been conducted on the role of melatonin in the development and treatment of many diseases, including AD, BA and AR which are atopic diseases with similar pathophysiology. In previous studies with murine models, melatonin was reported to reduce IgE, IL-4 and IFN-γ levels and severity of AD symptoms through its reduction of activated CD4+ T cells (13). Furthermore, it was shown to contribute to the control of asthma symptoms by reducing MMP-9 (19), TLR-9 (20) and MUC5AC (21). In addition, melatonin levels have been shown to decrease in AD (3), BA (22) and AR in clinical studies (23,24). Chang et al. demonstrated that 3 mg / day oral melatonin treatment was more effective than placebo in the control of AD symptoms in children (25). In a study evaluating the effectiveness of oral melatonin in lung inflammation and airway hyperactivity, it was determined that it shows anti-inflammatory activity by reducing eosinophil and neutrophil and TNFα level in Broncho-alveolar Lavage (BAL). Moreover, melatonin was reported to decrease NO and hydroxyl radical concentrations and iNOS, TNF-α, nitrotyrosine and myeloperoxidase (MPO) activity in lung tissue (26). Our findings indicated that melatonin treatment causes anti-oxidant and immunomodulatory activity by reducing COX-1, ROS, IDO mRNA levels and IL-6 expression for the first time. These findings indicate that melatonin may be a new therapeutic approach for the treatment of AR.

Nasal polyps are benign inflammatory masses that are considered as a subset of chronic rhinosinusitis, originating from the nasal mucosa and paranasal sinuses, and their frequency increases with age (27). In the pathophysiology of nasal polyps, similar to AR, there are type 2 inflammation patterns characterized by eosinophilia and high IL-4, IL-5 and IL-13 cytokine levels (28). Mainly intranasal glucocorticoids are used on the basis of nasal polyp treatment and prevention. In patients whose symptoms cannot be controlled by medical treatment, polypectomy is performed with endoscopic sinus surgery (29). However, relief of attacks of their symptoms after surgery may not be curative because the nasal polyps show high recurrence rates; therefore, indicating that the treatment of underlying mechanisms is crucial. In a prospective multicenter cohort study was evaluated that relapse rates of 363 adult patients who underwent endoscopic sinus surgery for polyposis, within 18 months were found to be around 40% (30). In our study, melatonin has been shown to have more prominent anti-inflammatory and anti-oxidant activity in the nasal polyp tissue. According to our results, Np-MSC's has higher response to melatonin treatment, thus we believe that the effects of melatonin on nasal polyp treatment should be assessed in future studies.

Considering the results of the study, it is predicted that some limitations may be encountered. The effects of melatonin on the serum levels of various cells, mediators and cytokines (which could influence inflammatory response) could not be evaluated. Therefore, additional animal and clinical studies are required to reveal the effectiveness of melatonin in AR and nasal polyposis.

In conclusion, for the first time, our study demonstrated that the effectiveness of melatonin treatment in AR and nasal polyposis. Our findings indicated that the anti-oxidant and anti-inflammatory activity of melatonin in experimental AR and NP cell culture models could be an important source of data for future in vivo and clinical studies. On the basis of these data, melatonin could be a promising agent for the treatment of AR and nasal polyposis.

Aknowledgement

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Conflicts of interest

No conflict of interest was declared by the authors.

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