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# Downregulation of IL-6 and TNF-α Expression with Mesenchymal Stem Cells Therapy in Allergic Rhinitis Rats Models

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## ABSTRACT

**Background:** Rhinitis is an inflammatory that is characterized by nasal symptoms due to the condition of the nasal mucosa triggered by an interaction between environmental allergens and immunoglobulin (Ig)E. It is driven by host factors, infection, pathogens, and various inflammatory pathways such as TNF-α that are released in allergic responses from both mast cells and macrophages through IgE-dependent mechanisms. Mesenchymal stem cells secrete various potent anti-inflammatory soluble molecules that we're able to improve the conditions of endothelial damage, inflammation and oxidative stress by decreasing levels of IL-6 and TNF-α.
**Objective:** This study purposed to investigate the effect of UC-MSCs on TNF-α and IL-6 expression in rat model ovalbumin-induced rhinitis in vivo.
**Methods:** Thirty male Wistar rats were randomly divided into 3 groups: control, treatment and sham groups that were administrated through intramuscular injection.
**Results:** Based on histological analysis, MSCs decrease the expression area of TNF-α and IL-6.
**Conclusion:** The results of this study found that UC-MSCs can downregulate the inflammatory cytokines TNF-α and IL-6 in rat model ovalbumin-induced rhinitis in vivo.

**Keywords:** Mesenchymal Stem Cells, TNF-α, IL-6, Rhinitis Allergy.

## INTRODUCTION

Rhinitis is an inflammatory condition of the nasal mucosa that is triggered by an interaction between environmental allergens and immunoglobulin E (IgE) in sensitized individuals[1]. It is characterized by nasal symptoms including congestion, sneezing, itching and rhinorrhea, as well as ocular effects such as eye itching, tearing and redness. Rhinitis has a fairly high prevalence, often found in most populations in the world which is around 7-30%[2–4]. The prevalence of rhinitis in China in 2021 amounted to 4.8-9.7% estimated 107 million population, in the United States in 2017 about 14% with an estimated 37 million population, and in Europe, it is estimated at around 7-27% in people adults [2,5]. In Indonesia, the prevalence of rhinitis has a range between 1,5-12.3%, Jakarta 26,71% and increases every year [6].

Rhinitis was thought to be simply a bacterial infection but, a recent study has revealed that inflammation plays a role important in persistent chronic rhinosinusitis[7]. Tissue biopsy results sinuses at surgery showed inflammatory cells and pro cytokines inflammation inactive disease, one of which is Tumor Necrosis Factor-alpha (TNF-α)[8–10]. This airway epithelium triggers the production of

proinflammatory cytokines in a large quantity. Previous clinical study with chronic rhinosinusitis and found pro-inflammatory cytokines, one of which is TNF-α in paranasal sinuses and conchae[12,13]. TNF-α is a proinflammatory cytokine produced by several cell types. TNF-α has been implicated in various inflammations, infections and malignancies. The management of rhinitis presents challenges for clinicians, at present, regular drug treatment could relieve the allergic symptoms, but could not interfere with the allergic reactions. There is evidence that rhinitis is driven by various inflammatory pathways and host factors, not just a matter of infection, although pathogens, including bacterial biofilms, may be able to contribute to the inflammatory cascade and treatment resistance.

Mesenchymal stem cells (MSCs) secreted enriched anti-inflammatory soluble molecules such as interleukin-10 (IL-10) interleukin-1 (IL-1) and growth factors such as transforming growth factor-(TGF-β), Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), and Hepatocyte Growth Factor (HGF)[14,15]. Conditioned medium MSCs were able to improve the conditions of endothelial damage, inflammation and oxidative stress by decreasing levels of IL-6, TNF-α, ROS and increasing eNOS. IL-10 released by MSCs can downregulate the production of inflammatory cytokines and inhibit T cell activation associated with the immune response[16–19]. IL-10 is also a potent inhibitor of Th1 pro-inflammatory cytokines such as IL-2 and IL-6 [20].

IL-6 is a pro-inflammatory cytokine and has been reported to be involved in the suppression of T cell proliferation and local inflammation. IL-6 is a pleiotropic and multifunctional cytokine involved in modulating various physiological events, such as cell proliferation, differentiation, survival and apoptosis[21,22]. Generally, the biological functions of IL-6 are achieved via the Janus-associated kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, the Ras–Raf–mitogen-activated protein kinase (MAPK) pathway and the PI3K/Akt pathway[23,24]. In the central nervous system (CNS), the IL-6 levels are low under normal conditions but are significantly elevated under disease conditions[25]. Both detrimental and beneficial functions of this cytokine have been reported. Therefore, in this study, we explore the potential of umbilical cord mesenchymal stem cells (UC-MSCs) as an alternative agent to suppress the expression of proinflammatory cytokines in rhinitis. This study purposed to investigate the effect of UC-MSCs on TNF-α and IL-6 expression in rat model ovalbumin-induced rhinitis in vivo.

## MATERIAL AND METHODS

### *MSCs isolation and characterization*

MSCs were isolated from the umbilical cords (UC) of pregnant rats. The umbilical cords tissues were flushed with Phosphate Buffer Saline supplemented with 2% fetal bovine serum (FBS). The umbilical cords tissues were plated and cultured at 37 °C temperature and 5% O2 in the Gibco Dulbecco's Modified Eagle's Medium Low Glucose supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 0.1 mg/ml streptomycin. The medium was replaced every 3 days, and the cells were sub cultured when confluency exceeded 90%. UC-MSCs from passages 3 to 5 were used for the studies. UC-MSCs were characterized according to criteria established by the International Society for Cell and Gene Therapy including the ability of MSCs to adhere to plastic surfaces, the potential for differentiation of MSCs into adipocytes (Oil red O staining), osteocytes (Alizarin Red S staining), and chondrocytes (Safranin O staining). Meanwhile, mouse MSC cell surface markers identified using flow cytometry showed positive values for CD90, CD29, CD54, CD73 and negative values for CD31, CD45, and CD34[19].

### *Animals*

Male Wistar rats (250–300 g) were obtained from the Animal Health Education and Training Unit Laboratory, Faculty of Veterinary, Gadjah Mada University. This study was approved by the Ethical Committee Universitas Sumatera Utara (142/KEP/USU/2020). All rats were housed under control temperature room (temperature: 24 ± 1°C, humidity: 50 ± 5%, and 12-h light/dark cycle), with free access to food and water.

### *Experimental design*

Thirty male rats were randomly divided into 3 groups ($n$=10): control group, treatment group, and sham group. All the animals except the sham group received repeated intranasal instillation of OVA in sodium chloride saline solvent with 300 µl for 3 consecutive days. The OVA antigen was purchased from Sigma, grade V, St. Louis, Missouri, (A8040, USA). The sham group was intranasally instilled with normal sodium chloride instead of the same procedure. In the treatment group, UC-MSCs were administrated with the indicated $3x10^6$ cells through intraperitoneal injection, once a day for 3$^{rd}$ days after being validated as rhinitis animal models. The number of sneezes, degree of runny nose, and nasal rubbing movements during the 30 min period after the final allergen challenge were recorded in each experimental group.

### *Specimen Collection*

Termination of rats through intramuscular injection of 10 mL cocktail, Ketamine 50 mg/kg BW, Xylazine 10 mg/kg BW and Acepromazine 2 mg/kg BW before organ harvesting. The murine nasal mucosa tissues were excised and fixed overnight in 10 % neutral-buffered formaldehyde at 20 C and then embedded in paraffin. The paraffin-embedded specimens were sectioned into 3um thickness on a microtome, mounted on slides and deparaffinized. After that, tissue sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS stain kit; ScyTek Laboratories, Inc., Logan, UT, USA) according to the manufacturer's instruction to detect the extent of mast cell infiltration.

### *Immunohistochemical (IHC) Analysis*

Primary antibodies of TNF-α and IL-6 were purchased from (Protein tech, Wuhan, China). Paraffin sections of hippocampus tissue were stained with the streptavidin peroxidase method to examine TNF-α and IL-6 expressions. The sample was deparaffinated by immersing the samples in xylene I II, and III each for 5 minutes followed by rehydration for 5 minutes in 100% ethanol II, 100% I, 90%, 80%, 70% and then washed in running water for 5 minutes. The next step was Antigen retrieval with citrate buffer 1x and incubation at $95^0$C for 5 minutes. After that, the samples were washed using PBS and continued with Endogenous Peroxidase Inhibition by adding 3% $H_2O_2$ solution 60-200 µl on the sample tissue with a micropipette for 5 minutes then slide was washed with PBS 1 x 1000 µl for 5 minutes 3 times. Blocking background was using background Sniper solution (Starr Trek Universal-HRP Detection Kit) on grid per slide. Give First Antibody (dilution 1:200) in each tissue 100 µl and incubated overnight. The next day, after incubation, the tissue slides were washed again using PBS.

The next step is the incubation of the sample with Trekkie Universal Link secondary antibody (Starr Trek Universal-HRP Detection Kit) on tissue per slide of 100 µl for 90 minutes at room temperature. After that, samples were washed using PBS and continued with the addition of 70-100 µl DAB per slide then incubated in a dark room for 5 minutes and then washed again. The next step was staining using hematoxylin for 5 minutes. Put the rack in a glass jar, and wash in running water for 5 minutes. The last stage is dehydration and clearing in xylene. Mounting with entellan drip and closure with glass deck. The stained sections were observed in mounting media for image acquisition. The cells were analyzed by microscopy at 400x magnification, and the TNF-α and IL-6 positive cells were

quantified by the average number of positively stained cells per field. Mast cells in the nasal mucosal sections were counted and recorded under a light microscope (Olympus). Six different sections of each mouse were calculated. The average number of mast cells per high-power field (n/hpf) in each mouse was compared and analyzed densitometry by ImageJ software, version 1.46r (National Institutes of Health, Bethesda, MD).

### *Statistical Analysis*

Data were represented as mean ± standard deviation (SD) (SPSS statistical analysis software, version 20.0). All variables were analyzed of normal distribution, homogeneity test and parametric testing one-way analysis of variance (ANOVA), followed by post hoc analysis using the Bonferroni test. Differences were considered to be statistically significant at a p-value $< 0.05$.

## RESULTS

### *MSC characterization and differentiation assays*

The MSCs culture after the fourth passage showed cells adhering to the bottom of the flask with spindle-like cell morphology under microscopic observation (Figure 1A). The adipogenic differentiation assay was performed by inducing MSCs using a special adipogenic medium that formed adipocytes (Figure 1B). The osteogenic differentiation assay was performed by inducing MSCs using a special osteogenic medium that formed osteocytes (Figure 1C). Flow cytometry analysis showed that MSCs were able to express CD90 (99.8%), CD29 (94.2%), and lack of CD45 (1.6%) and CD31 (6.6%) (Figure 1D).

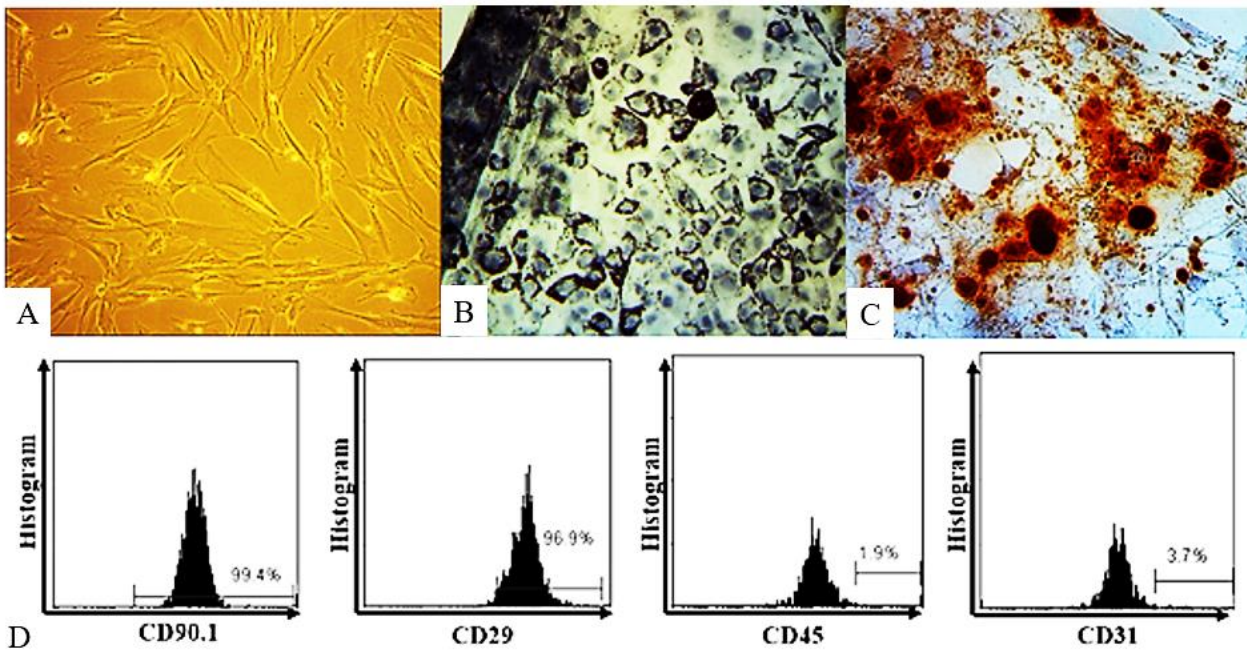


**Figure 1.** MSCs Validation and Differentiation Assay. Validation assay of MSCs morphology were fibroblasts like-cells at a microscope magnification of 50µm (A). It appears that MSCs are attached to the plastic surface with 80% confluency. MSC validation assays adipogenic differentiation: 50µm magnification (B). MSCs validation assay for osteogenic differentiation; 50µm magnification (C). MSC is shown by arrows on cells containing accumulated lipid droplets with Oil Red O staining. MSC validity was indicated by positive values at CD90.1 and CD29, while negative values at CD45 and CD31 (D). Quantitatively in the form of percentage expression CD.90.1 99.4% and CD29 96.9%.

### *MSCs decrease histological area expression of TNF-α and IL-6 in AR rats*

Histological examination of the three groups showed similar results. Sections of sham and treatment groups revealed normal nasal cavity mucosa with respiratory epithelium (pseudostratified columnar ciliated with goblet cells). The cells were tall columnar cells with some invaginations mostly to increase surface area resting on thin basal lamina and lamina propria of loose connective tissue containing multiple blood vessels and seromucous glands. Branching seromucous glands were noticed, reflecting the high secretory functions of these glands. The control group showed damaged respiratory epithelium (the epithelial cells lost their positions, mucosa exfoliation) with a focal area of epithelial hypertrophy resting on the thick basal lamina and lamina propria with infiltration of numerous cells (lymphocytes, eosinophils and plasma cells) and few blood vessels with seromucous glands. In the treatment group, there was partial damage of respiratory epithelium resting on the thick basal lamina, and the lamina propria had infiltration of a few inflammatory cells with few blood vessels. (Figure 2A).

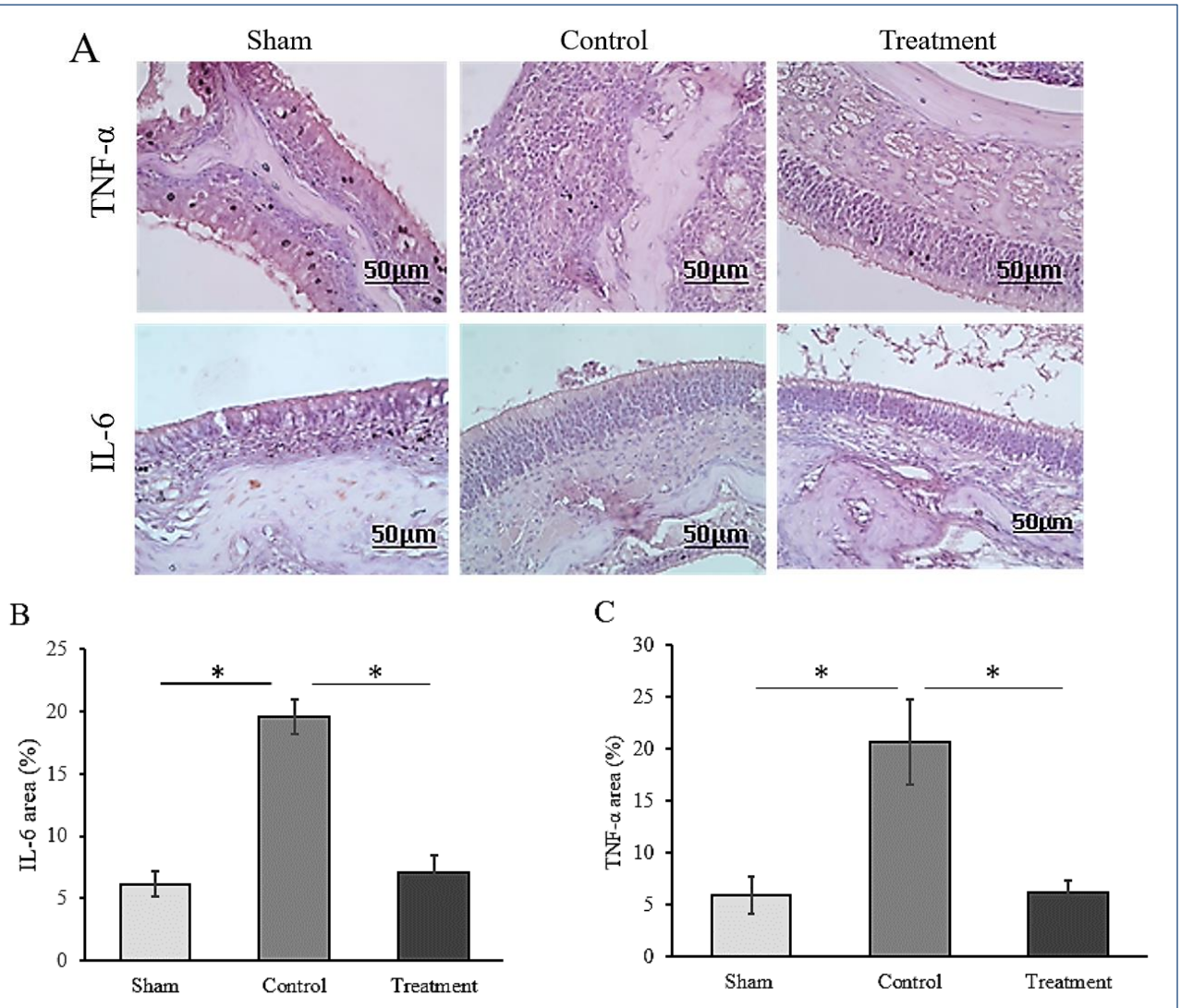


Figure 2. Photomicrograph of a section in the nasal mucosa of TNF-A detection sham, control and treatment group displaying comparison nasal mucosa with respiratory epithelium resting on thin basal lamina and lamina propria of loose connective tissue having multiple blood vessels and seromucous glands. A photomicrograph of a section in the nasal mucosa of AR group IL-6 assays sham, control and treatment group showing impairment of respiratory epithelium resting on the thick basal lamina and lamina propria with infiltration of abundant cells and with few blood cells. (50µm magnification) (A). Quantification of TNF-α expression in the nasal cavity tissue in three groups used the immunohistochemical staining method with TNF-α antibodies (B). Quantification of IL-6 expression in the nasal cavity tissue in three groups used the immunohistochemical staining method with IL-6 antibodies (C). The TNF-α and IL-6 expression in the control and treatment group on day 3 showed a significant decreased; *p<0.05.

Based on the observation of TNF-α expression on the third day, the number of mast cells in the treatment group showed a significant decrease ($p<0.05$) compared to the control group, 6.23±1.14 and 20.68±2, respectively. On the other hand, IL-6 expression of the treatment group also reduced to 7.119±1.3 compared to the control group, 19.533±1.3. Both variable expressions on the third day of observation in the treatment group had a lower number than that of the control rats' group (Figure. 2B and 2C).

## DISCUSSION

The pathophysiology of nasal congestion involves several mechanisms, including mucosal inflammation, venous dilation, increased nasal secretions, tissue swelling/edema; and/or modulation of sensory perception[1,4]. Many inflammatory mediators cause exudation plasma and vasodilation, with edema and swelling of the nasal mucosa.[26] Nowadays, chemical drugs for AR are limited to antihistamines, antileukotrienes, and intranasal corticosteroids, which only alleviate allergic symptoms but fail to regulate the allergic reaction[1].

MSCs are increasingly being found to have potent anti-inflammatory effects in a wide range of inflammatory and immune-mediated disease models[27,28]. Therefore, the immunomodulatory function of MSCs makes them promising candidates for allergic disease therapy. Administration of MSCs can ameliorate the severity of acute injury and fibrosis with modulation of pro-inflammatory and anti-inflammatory cytokines, which is considered the main beneficial effect of MSCs[29]. Since AR is characterized as chronic inflammation with eosinophilic infiltration and imbalances between TH1- and TH2-derived cytokines, we propose that MSCs-driven immunomodulation contributes to attenuation of inflammation in AR, consequently improving patient lifestyle[30].

The results of this study are following previous research found an increased TNF- α expression in chronic rhinosinusitis in animal model and patients. Recently, a previous study also found increased expression of TNF-α, IFN-Ɣ, and TGF-β1 in chronic rhinosinusitis without polyps[31]. Ebrahim, et al. (2019) stated that the expression of IL-1β and TNF-α in the nasal mucosa of a murine model with chronic bacterial rhinosinusitis was significantly increased compared to controls[8]. Under these results, Sun and his colleagues revealed that administration of Montelukast alone in a guinea pig model for allergic rhinitis (AR) failed to prevent the allergic rhinitis symptoms while with the combination therapy of Montelukast and sodium cromoglycate, the AR symptoms (sneezing and rubbing frequencies) were significantly prevented with marked prevention of inflammatory cells infiltration into the nasal mucosa [8].

TNF-α is produced from a variety of cell types. Although the main sources of TNF-α production are macrophages and monocytes, a variety of cells can produce TNF-α including T and B lymphocytes, mast cells, NK cells, endothelial cells, smooth muscle cells, and fibroblasts[22]. TLRs function as first responders in mammalian hosts to recognize invading pathogens and will be bound by TLRs. TLRs are found on APC cells and are required for Th0 cell activation[32]. The Th0 cells differentiate into Th1 cells which are macrophage stimulators. Macrophages will release cytokines, one of which is TNF-α. Min et al. (2017) revealed that despite differences in the literature regarding cytokine expression in patients with sinusitis, there is evidence that proinflammatory cytokines play a strong role in initiating and sustaining inflammation[3]. There was consistent overexpression of the four major inflammatory pathway genes (IL-6, IL-12A, IL-13, and TNF-α) in patients with chronic rhinosinusitis compared with the normal population[13]. The synergy of TNF-α plays an important role in the pathogenesis of conditions associated with inflammation, damage, and limited tissue repair.

## CONCLUSION

UC-MSCs downregulate the inflammatory cytokines TNF-α and IL-6 in rats model ovalbumin-induced rhinitis in vivo.

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**AUTHORS' CONTRIBUTIONS**

LR builds the conception and design of the study and acquisition of data, SSG and EH analysis and interpretation of data, MAN and RCSI drafting the article or revising it critically for important intellectual content and final approval of the version to be submitted.

**COMPETING INTERESTS**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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