Effect of interferon-γ on the expression of regulatory *NLRP6* and *NLRP12* genes in human Wharton's jelly mesenchymal stem cells

Running title: IFN-induced NLR gene expression

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Abstract

Background: Mesenchymal stem cells (MSCs) are well known as a major immune modulator. A subgroup of the nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) has been recently found to play an immune/inflammatory regulatory role.

We aimed to analyze and compare the gene expression levels of the NOD- like receptor family pyrin domain- containing proteins (NLRPs), such as NLRP6 and NLRP12, in Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) treated with interferon-gamma (IFN-γ), the pro-inflammatory cytokine, and untreated cells as well.

Methods: The immunophenotypic characterization of the isolated WJ-MSCs was performed by flow cytometry. Next, they were cultured with or without IFN-γ, followed by the comparison of expression level of NLRP6 and NLRP12 genes by using qPCR.

Results: The treatment of cells with IFN-γ indicated a statistically significant increased expression of NLRP12 gene as compared to untreated cells while the expression of NLRP6 did not differ significantly between cells with or without IFN-γ treatment.

Conclusion: The altered expression level of NLRP12 might be suggested its contributory role in the inflammatory regulation mediated by WJ-MSCs in response to the exposure to IFN-γ; however, additional studies are needed to validate its role in experimental inflammatory-related disease models.

Keywords: Gene Expression, Interferon-gamma, Inflammation, Mesenchymal stem cells, NOD like receptors

Introduction

Mesenchymal stem cells (MSCs) are well considered as the suppressor of immune/inflammatory responses, indicating their potential application in cell-based therapies (1, 2). Wharton's jelly MSCs (WJ-MSCs) possess lower immunogenicity related to both reduced expression of MHC and co-stimulatory molecules [B7-1 (CD80) and B7-2 (CD86)] (3, 4). This supports the development of MSC-based therapy under inflammatory conditions (5). The MSC-mediated immunomodulation occurs following the interaction of MSC with immune cells and their secretions, such as inflammatory cytokines (6). Interferon-gamma (IFN-γ) can regulate immune/inflammatory responses in both health and disease (7-9). The cytokine is necessary to either license or improve the immunosuppressive functions of MSCs, since MSCs-mediated suppression of cell proliferation and function has been reported in the presence of IFN-γ (10-12). The innate immunity is capable of determining MSC characteristics. Recently, the findings have shown the induction of pro-inflammatory phenotype of MSC1 and the anti-inflammatory phenotype of MSC2 (13). Nucleotide-binding oligomerization domain receptors (NOD-like receptors, NLRs), as key mediators of inflammatory responses, enable to identify pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern (DAMPs) (14, 15). The inhibitory NLRs, in particular NLRP12 and NLRP6, is thought to negatively regulate the nuclear factor NF-κB pro-inflammatory signaling pathway, resulting in the attenuation of inflammatory response (16-18). This is attributed to the downregulated expression of IFN-related genes, induction of M2 macrophages-mediated immunosuppressive microenvironment, and promotion of $P14^{ARF}$–Mdm2–P53-dependent cellular senescence (19-22). Of note, an upregulation of the NLRPs plays a role in the maintenance of HSCs (23). Furthermore, their negative regulatory functions have been reported during bacterial infections like *L.monocytogenes* and *B.abortu,* which is mediated through the dysregulation of NF-κB activation and increased production of pro-inflammatory cytokines (24, 25). Therefore, it is important to focus on the regulation of innate immune and inflammatory responses. In the present study, we aimed to evaluate the gene expression level of NLRP6 and NLRP12*,* known as possible negative regulators of inflammatory signaling, in WJ-MSCs treated with IFN-γ mimicking an inflammation scenario by real-time qPCR. Appropriate modulation of innate immune mechanisms may be permissive for the establishment and maintenance of immunosuppression in the inflammatory milieu.

Method

Isolation and culture of Wharton's jelly MSCs

The cells isolated from the umbilical cord in a study which had the approval of the institutional local ethics committee (IR.IAU.TABRIZ.REC.1398.029) was used for further evaluation in this study. This research was conducted at the Islamic Azad University of Ardabil Branch in 2021. After obtaining informed consent, the umbilical cords collected from full-term births after cesarean section and/or normal vaginal were transferred to the laboratory in normal saline and the cells were isolated by using a conventional tissue culture procedure. Briefly, the cords were cut into 2 cm pieces soaked in Hanks' balanced salt solution (HBSS) and WJ-MSCs was chopped into small pieces after removal of the vessels. Cells were then cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Germany) supplemented with 20% fetal bovine serum (FBS) (Gibco, Germany), 1% penicillin/streptomycin (Sigma, USA), and 1% amphotericin B (Sigma, USA). They were incubated at 37°C in 5% CO2 humidified atmosphere, followed by the examination of cell cultures every three days with an inverted microscope to check the

possibility of contamination and their maintenance for 10 days in an incubator. Following the identification of first mesenchymal stem cell, the renewal of media was performed every three days to reach 70-80% confluence (26). Subsequently, the cells were harvested using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, UK) and then passaged to new flasks.

Flow cytometry analysis in Wharton's jelly stem cells

Immunophenotyping of the isolated MSCs was performed by flow cytometry. To perform this, cells were dissolved in a stain solution, and a cell suspension with $1\text{-}2\times10^6$ cells/mL was prepared. Antibodies against MSC surface markers CD34, CD44, CD45, CD73, CD90, and CD105 were then added to each tube according to the manufacturer's protocol, followed by an incubation period. Isotype antibodies were used to rule out the background fluorescence using the same procedure. The cells were finally analyzed using a FACSCalibur flow cytometer (BD Biosciences, USA), and FlowJo software (version 7.6.1) was used for subsequent analysis.

RNA isolation and cDNA synthesis

Wharton's jelly-derived MSCs (0.5×10^6 cells/mL) were divided into two groups; one group was treated with IFN-γ (20 ng/mL) for 24 hours, and the other remained untreated (27). Note, a short-term exposure to the pro-inflammatory cytokine IFN-γ (as mimicked by 24-hour licensing) has been documented to enhance immunomodulatory capacities of cultured human MSCs, but did not induce cell apoptosis (28). Subsequently, total RNA was extracted using the One-Step RNA Reagent (Bio Basic) kit according to the manufacturer's instructions. Treatment with DNase I (Thermo Fisher Scientific, USA) was also performed to remove genomic contamination from the extracted RNA samples. The ratio of absorbance at 260 and 280 nm was used for purity assessment of RNA samples. RNA quality was also assessed using agarose gel electrophoresis. These RNA samples were reversely transcribed into cDNA (Complementary DNA) using the PrimeScript™ RT Reagent Kit (Takara, Japan) according to the manufacturer's protocol. A polymerase chain reaction (PCR) amplification for each target genes was carried out in triplicate wells in which the reaction mixture (total volume 20 µl) contained 12.5 µl of master mix, 1 µl of each forward and reverse primers, 1µl of cDNA, and 4.5 µl sterile distilled water under following conditions: 10 minutes at 95ºC followed by 35 cycles of 40 seconds at 95ºC, 30 seconds at 60ºC, and 30 seconds at 72ºC, with 5 minutes at 72ºC for final extension. The same PCR condition was used for amplification of the NLRP6 and GAPDH genes except that the annealing temperature was 63ºC and 59ºC, respectively. A negative control containing no template was used as well. The amplification products were analyzed by 1.5% agarose gel electrophoresis and visualized by safe nucleic acid staining reagent.

Real-time quantitative PCR (qPCR)

The mRNA expression levels of target genes in the treated and untreated samples were quantified by qRT-PCR using the SYBR Premix Ex Taq II kit (Takara, Japan) according to the manufacturer's procedures. The real-time PCR was performed in a final volume of 20 µl containing 10 µl of SYBR master mix, 0.5 µl of each forward and reverse primer, and 2 µl of template cDNA. The cycling conditions were 95°C for 10 minutes followed by 45 cycles of 95°C for 10 seconds, an annealing temperature (varies for different genes) for 30 seconds, and 72°C for 20 seconds. The melting temperature cycle was performed from 55°C to 95°C to generate a melt curve chart for confirmation of product specificity. Duplicate reactions were run for each gene in the Rotor-Gene 6000 thermal cycler (Qiagen, Netherlands). The cyclic threshold (CT) values were used to analyze the expression levels via the $2^{-\Delta\Delta Ct}$ method. The expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts was measured and used

for normalizing the gene expressions. The same procedure was also used for a negative template control. The primer sequences used in this study are listed in Table 1.

Statistical analysis

To statistically analyze the data, the GraphPad Prism software (version 5.04) was used. The results were analyzed using Mann–Whitney tests and presented as mean ± SD. A value of $P<0.05$ was considered statistically significant.

Result

Morphological and immunophenotypic characteristics of WJ-MSCs

After the WJ-MSCs pieces were plated and cultured for approximately 10 to 14 days, the stromal cells with fibroblast-like appearance were dissociated around tissue sections, which had been adhered to the culture flask (29). In the following week, these cells multiplied and grew around the adherent tissue pieces after the removal of tissue pieces, as the tissue culture flask was covered. They were examined with an inverted microscope during the culture period. The cells were then divided into several flasks when 80% confluence was achieved. The MSCs grown from the primary culture of human umbilical cord blood are indicated in Figure 1. Flowcytometric analysis showed the expression of common MSCs markers CD44, CD90, CD105, and CD73 on the isolated cells, whereas no expression of CD34 and CD45 markers was observed in these cells (Figure 2).

Real-time qPCR results

After cDNA synthesis, the cDNA was amplified with specific primers for NLRP12, NLRP6 and GAPDH by RT-PCR. The desired bands were observed in the 250 bp, 181 bp and 218 bp on gel electrophoresis, respectively (Figure 3). Expression levels of NLRP6 and NLRP12 mRNA transcripts were analyzed using qRT-PCR in either untreated WJMSCs (control) or WJMSCs treated with IFN-γ after 24 hours. Expression levels of the target genes were normalized against GAPDH mRNA expression as housekeeping gene, and the normalized values were calculated using $2^{-\Delta\Delta Ct}$ method. As it is indicated in Figure 4, while no difference in expression levels of NLRP6 was shown between untreated and IFN-γ-treated cells, the cells treated with IFN-γ cytokine were shown to be highly enriched with *NLRP12* mRNA transcripts compared to the untreated control group ($P\leq 0.05$).

Discussion

The immunosuppressive capacity of MSCs make them a promising alternative immune/inflammation modulatory therapeutic approach (28). Considering the importance of NLRs in the inflammatory response, we were interested in evaluating the expression level of negative regulators of NF-κB signaling pathway, such as NLRP6 and NLRP12, following the treatment of MSCs with the pro-inflammatory cytokine IFN-γ. Interestingly, IFN-γ pre-licensing enhances immunomodulatory capacities of cultured human MSCs in inflammatory conditions (29). However, the underlying effector mechanisms of MSCs generating a suitable immune modulatory microenvironment needs to be investigated.

MSCs modulate the host immune responses through various mechanisms (30). Nicola *et al*. (11) have reported dose-dependent anti-proliferative effect of MSCs on T lymphocytes in the mixed lymphocyte culture (MLC) response. The NLRs participate in key mechanisms of immune responses, including antigen presentation (NLRC5) and the modulation of inflammation (NLRC3, NLRP6, NLRP12, NLRX1) (22). The NLRP12 has been indicated to negatively regulate T cell responses and IL-4-associated inflammation in experimental autoimmune

encephalomyelitis (EAE) disease (31, 32). In another study, NLRP6 deficiency is found to increase an inflammation and induce tumorigenesis (33).

In this study, the expression level of NLRP6 and NLRP12 was evaluated in MScs isolated from Wharton's jelly of the human umbilical cords after treatment with IFN-γ, since they can regulate an inflammatory signaling. It was observed that the expression level of NLRP12 in the WJ-MSCs treated with IFN-γ was higher than in the control group, while the treated group exhibited no difference in *NLRP6* expression. This might be resulted from the low dose of INF-γ or insufficient of the treatment duration. When considering the dominant role of IFN-γ in MSCs licensing, it is necessary to adjust the levels of this cytokine in the setting of an inflammatory response (34, 35). Another possibility is that INF-γ by itself may be incapable of mimicking the *in vivo* inflammatory condition to remarkably induce the NLRP6 gene expression. Moreover, the NLRP6 expression can be inhibited through miRNA (36). Thus, the RNA and/or protein products of other genes may serve to regulate its expression.

The NLRP12 has a regulatory function in the innate immune activation in type I interferon (IFN-I)–dependent manner, as demonstrated by its overactivation under low NLRP12 expression (37). Stimulation with IFN-γ plus TNF has been shown to regulate PANoptosis (characterized by pyroptosis, apoptosis and necroptosis) by modulating NLRP12 expression (38). Other study has revealed that INF-γ priming and NLRP12/NLRP3-dependent activation of caspase 1 mediate hypersensitivity to secondary bacterial infection during malaria (39). In addition, the NLRP12 suppresses NF-κB and ERK activation associated with the inhibition of inflammatory cytokines and nitric oxide production in tumorigenesis (30, 40). Despite the role of NLRP6 deficiency in the reduction of IFN-γ production and augmentation of caspase-1 activation (41), its overexpression may lead to the attenuation of cell proliferation (21). Presumably, unchanged NLRP6 expression in treated MScs might serve a protective role in the maintenance of MSC survival; however, its effect on the capability of modulating inflammation needs to be clarified. Of note, their regulatory mechanism and additional functions should be investigated using different experimental settings. The IFN-γ mimicking an inflammatory environment may contribute to the enhanced gene expression in treated WJ-MSCs. However, multiple factors can play a role in determining either attenuation and/or activation of inflammation. In this regard, the findings of our study showed the expression of *NLRC3* and *NLRC5* genes at high levels in IFN-γ-treated WJ-MSCs as compared to untreated cells (42).

This study provides insight into new gene targets identification involved in the modulating activity of MSCs. Nevertheless, the modulatory role of MSCs expressing NLRs on the function and regulation of innate immune signaling pathways needs to be evaluated under various in vitro and in vivo inflammatory conditions.

Conclusion

Regarding the reported anti-inflammatory effects of a subgroup of NLR family, this study demonstrated the modulatory effect of pro-inflammatory cytokine IFN-γ, known as a major player during a variety of pathological situations and tissue injury, on the expression level of NLRP12 in the treated MSCs. However, subsequent research is required to examine its functional association with the underlying mechanisms probably involved in MSC-mediated modulation in dose- and/or time-dependent manners.

Acknowledgment

This work was financially supported by Islamic Azad University, Tabriz Branch, Iran. We also wish to thank all the individuals at Islamic Azad University, Ardabil Branch, Iran, and Sabalan Hospital who assisted us in this research.

Author contribution

All authors have contributed equally.

Funding sources

This work was financially supported by Islamic Azad University, East Azarbaijan Branch, Tabriz, Iran.

Ethical statement

The project was approved by the institutional local ethics committee (IR.IAU.TABRIZ.REC.1398.029).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors have no conflicts of interest to declare.

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Table 1. Primer Sequences used for RT-PCR and qPCR assays

Gene	Primer sequence (5'- 3')	Size (bp)
NLRP6	F: GCATGGACGTGGCTGTTCT R: GCTGGCAGTTGTTTTGTGGT	181
NLRP12	F: TTACCTGACCAACAACGCC R: CAGCAGCCAATGTCCAAAT	250
GAPDH	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	218

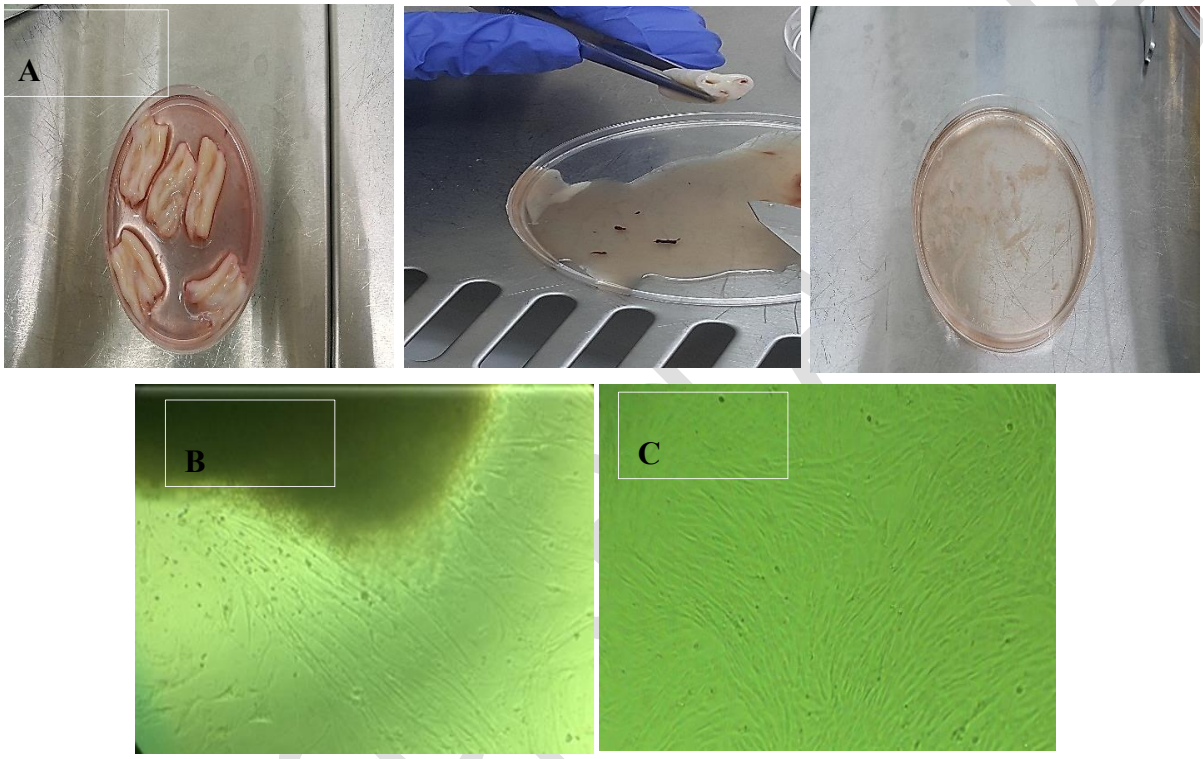

Figure 1. Human umbilical cord dissection and isolation of cells; minced human umbilical cord tissue was then cultured to release mesenchyme stem cells. A: The Morphological characteristics of MSCs isolated from WJ-MSCs. B: Cell buds observed after 10-14 days of primary culture growing around the WJ-MSCs tissue section (40×). C: After the cells were passaged, the adherent cells with fibroblast-like appearance exhibited a high proliferative ability and covered the culture flask (40×).

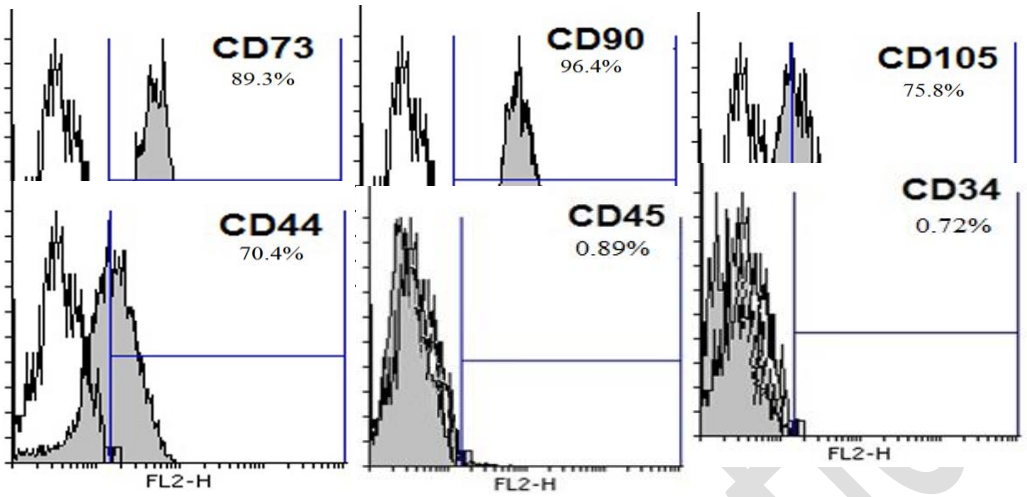

Figure 2. Immunophenotyping results of WJ-MSCs. White-filed histogram and grey-filled histogram represent fluorescence intensity (log) of cells stained with isotype control and specific antibodies, respectively.

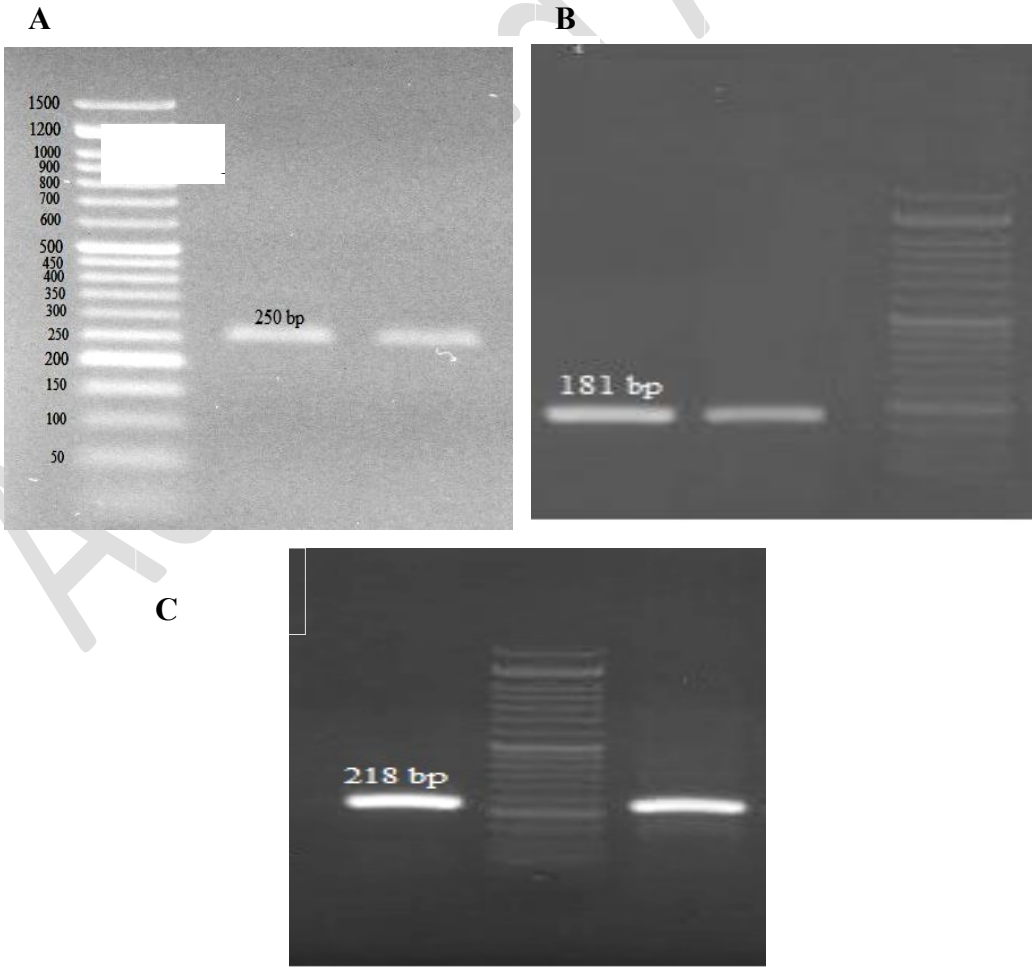

Figure 3. Gel electrophoresis results of the RT-PCR product of the WJ-MSCs. A: NLRP12 gene, B: NLRP6 gene, C: GAPDH gene.

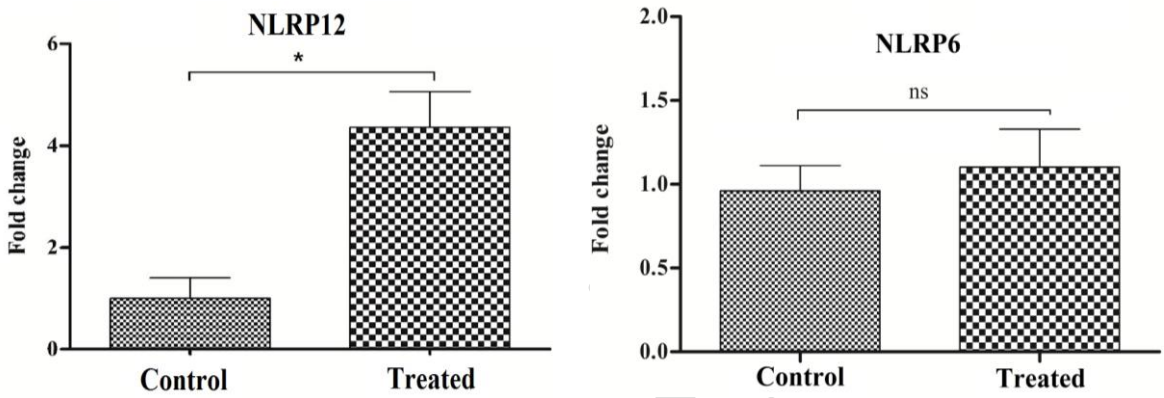

Figure 4. NLRP12 and NLRP6 gene expression in both untreated and IFN-γ-treated WJ-MSCs. *; P≤ 0.05, ns; not significant.