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Long-Term Treatment Effects of Low-Dose Naltrexone on Immunomodulatory Properties of Human Adipose-Derived Mesenchymal Stem Cells

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ABSTRACT

Background: Low-dose naltrexone (LDN) is involved in the treatment of inflammatory and immune system diseases and can affect immune cells. Mesenchymal stem cells (MSCs) are known for their immunomodulatory effects and the potential for the treatment of certain types of autoimmune diseases.

Objective: To investigate the long-term effects of LDN on human adipose-derived mesenchymal stem cells (ASCs) to see how their immunomodulatory properties are affected and also how LDN-treated ASCs interact with other immune cells present in peripheral blood mononuclear cells (PBMCs).

Methods: After 14 days of treatment, the ability of LDN-treated ASCs to modulate PBMC proliferation in a two-way mixed lymphocyte reaction (MLR) model was assessed using XTT. The relative expression of IDO, PD-L1, COX-2, HGF genes, and the level of IL-6 and TGF-β cytokines were measured in IFN-γ stimulated and unstimulated ASCs (treated and not treated cells) using real-time PCR and ELISA respectively.

Results: Unstimulated ASCs treated with 10-8 M Naltrexone (10-8 M NTX) showed higher levels of TGF-β, compared with the controls ($P<0.05$). Stimulated ASCs treated with 10-6 M NTX showed elevated expression of IDO, PD-L1 genes, and IL-6 level ($P<0.05$).

Conclusion: Our results demonstrated that various LDN concentrations have dissimilar effects on ASCs' immunomodulatory properties. A higher LDN concentration induced an alteration in the immunomodulatory features of ASCs.

Keywords: Adipose-Derived Mesenchymal Stem Cells, Immunomodulation, Inflammatory Response, LDN, Naltrexone

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INTRODUCTION

Mesenchymal stem cells (MSCs) are cells of mesodermal origin isolated from various tissues, including bone marrow, fat, placenta, and amniotic fluid. MSCs are defined by their capability to adhere to plastic and differentiation into several lineages such as adipogenic, osteogenic, and chondrogenic (1). The immunomodulatory ability of MSCs is acknowledged and therefore they are used in the treatment of autoimmune diseases such as multiple sclerosis (MS) and Crohn's disease (2). MSCs' therapeutic effects are carried out through an array of immunomodulation factors like prostaglandin E2 (PGE2), transforming growth factor-β (TGF-β), Interleukin-6 (IL-6), hepatocyte growth factor (HGF), and indoleamine 2,3-dioxygenase (IDO) (3).

Naltrexone hydrochloride (NTX), is an antagonist of mu-, delta-, and kappa-opioid receptors, commonly used in opioid addiction treatments (4). Low-dose naltrexone (LDN) temporarily blocks opioid receptors, resulting in an increased expression of receptors and levels of central and circulating endogenous opioids such as beta-endorphins and met-enkephalins (5). The diverse effects of LDN have been demonstrated in previous investigations. For instance, the administration of LDN reduces the proliferation of neuroblastoma cells in mice, but high doses of naltrexone increase the proliferation of these cells (6).

The immunomodulatory role of LDN is also of particular interest. Studies on the effect of LDN on macrophages and DCs showed that LDN could increase the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), IL-1β, IL-6, and IL-12. It also enhances the function and maturation of these cells (7, 8). On the other hand, the anti-inflammatory activity of LDN in mouse models of inflammatory bowel disease (IBD) and experimental autoimmune encephalomyelitis (EAE) deteriorates the level of pro-inflammatory cytokines such as TNF, IL-6, and IL-12 and suppresses B and T lymphocytes proliferation in these models (9, 10). Prolonged prescription of LDN has been effective in the remission of immune-related diseases such as MS (11), complex regional pain syndrome (CRPS) (12), Crohn's disease (13), and Fibromyalgia (14). Opioid antagonists' effect on immunomodulatory features of MSCs has not been studied yet, however, previous studies have indicated the effect of opioids on these cells. Bone marrow-MSCs (BM-MSCs) treatment with morphine decreases osteogenic and adipogenic differentiation as well as the proliferation of these cells in a dose-dependent manner. Moreover, higher concentrations of morphine increase the expression of IDO, cyclooxygenase-2 (COX-2) , and programed death-ligand 1 (PD-L1) immunomodulatory-related genes in these cells (15).

The immunomodulatory properties of MSCs propound them as important performers in the determination of the immune response outcome. Also taking into consideration the effect of opioids and their antagonists on immune system cells and MSCs as well as the application of opioid antagonists (naltrexone) in the treatment of inflammatory and autoimmune diseases, in this study, we aimed to investigate the effects of long-term LDN treatment in different concentrations on the immunomodulatory properties of human adipose tissue mesenchymal stem cells (ASCs).

MATERIALS AND METHODS

Isolation and Culture of ASCs

Visceral adipose tissue samples (N=3) were isolated from healthy women undergoing laparoscopic surgery (without underlying the health conditions associated with adipose tissue, aged 25-40 years) after obtaining written informed consent. The samples were transferred to the laboratory in Hanks Basic Salt Solution (HBSS) buffer containing streptomycin(300µg/ml),penicillin(300u/ml), and amphotericin B (25 µg/ml). After washing

with PBS buffer, the samples were cut into 5-mg pieces and placed in 6-well plates (SPL, Korea). Each piece was covered by 20 µl fetal bovine serum (FBS) (Life Technologies, UK). The plates were incubated for 24 h in a humidified atmosphere at 37 °C and 5% CO2. The following day, low glucose Dulbecco modified Eagle's medium (DMEM-LG) (Gibco, UK) containing 100 µ/m penicillin, 100 µg/ml streptomycin, and 15% FBS (complete medium) were added to the wells. The samples were cultured in the complete medium until spindle-shaped fibroblast cells appeared. The media were replaced every 48 h until the isolated cells reached 80% confluency. The cells were then detached using trypsin-EDTA 0.25% and cultured in T25 flasks. The cells were expanded until passage 4 for further experiments.

Characterization of MSCs

To confirm the stemness and mesenchymal nature of the cultured cells, their surface markers and multiple lineage differentiation capacities were examined. For the assessment of surface markers, the cultured cells at the 4th passage were trypsinized and washed with FACS buffer (PBS containing 0.1% BSA), and were incubated in dark at 4°C with CD90-Peridinin Chlorophyll Protein Complex (PerCP), CD105-isothiocyanate fluorescein (FITC), CD73-phycoerythrin (PE), CD45-FITC and CD34-PE (BioLegend, USA) anti-human conjugated monoclonal antibodies for 45 min. Irrelevant conjugated mouse antibodies with PerCP, PE, and FITC (BioLegend, USA) were also used as isotype controls. The cells were subsequently fixed using 4% formaldehyde. Finally, the expression status of the mentioned markers was assessed by FACS Calibur flow cytometry (Becton Dickinson, USA) and the results were analyzed by FLOWJO V.7.6 software. Moreover, to assess three lineage differentiation abilities of the isolated cells, the 4th passage was cultured with specific differentiation media for adipogenic, osteogenic, and chondrogenic lineages and stained specifically according to the method that we have described previously (16).

ASCs Treatment and Stimulation

To evaluate the effect of long-term LDN treatment on the immunomodulatory properties of ASCs, the cells from the 4th passage were treated with 10^{-8}, 10^{-7}, and 10^{-6} M of NTX (Jaber Ebne Hayyan Pharmaceutical Company, Iran) in separate groups for 14 days. As an explanation for the rationale behind considering 14 days of LDN treatment as long-term, it should be mentioned that this period was the longest time that the cells could be cultured without any obvious morphological modifications. During this period, the media was replaced every 3 days with complete media containing the desired doses of LDN. If necessary, the cells were passaged during this time. Untreated 14-days-cultured ASCs were used as the control. It was previously pointed out that the immunomodulatory properties of MSCs enhance under stimulated conditions, and one of the most conventional and important factors is IFN-γ (17). To assess the immunomodulatory features of the treated cells under stimulated conditions, the cells were washed with PBS on day 14 and cultured for 24 h in complete media containing 100 ng/ml IFN-γ (Exir Pharmaceutical, Iran).

Co-culture and Mixed Leukocyte Reaction

To evaluate the effect of unstimulated LDN-treated ASCs on the proliferation of peripheral blood mononuclear cells (PBMCs), a two-way mixed leukocyte reaction (MLR) was designed. In this regard, 5000 ASCs were seeded into a 96-well plate and treated with 10 µg/ml mitomycin C for 2 h (Biochem Pharmaceutical Industries Ltd). After this, the wells were washed five times with DMEM containing 10% FBS and incubated overnight. PBMCs were then isolated from two healthy volunteers using a Ficoll concentration gradient (1.077 g/ml) (GE Healthcare, Life Sciences, UK). Two-way MLR/ASCs co-culture was performed by adding 5×10^4 allogeneic PBMCs from each

individual to ASCs containing the wells. The two-way MLR/untreated ASCs co-culture was considered as the control. We would also like to mention that only unstimulated ASCs were used in this experiment to prevent the recurrence of IFN-γ impact since IFN-γ is produced throughout the MLR process. The proliferative response of allogeneic PBMCs on Days 0, 2, and 4 of co-culture was assessed using XTT colorimetric method (PromoCell GmbH, Germany) according to the manufacturer's instructions. Experiment well O.D.s on the mentioned days were read with an absorbance microplate reader (Stat Fax 2600, USA). Proliferation results were reported as normalized proliferation ratio that was calculated using the following formula and the normalized proliferation ratios of LDN-treated co-cultures were ultimately compared with the control group.

Normalized proliferation ratio=1-(O.D. of relevant day/O.D. of day 0)

RNA Extraction and Relative Reverse Transcription Real-time PCR

To evaluate the expression of COX-2, PD-L1, IDO, and HGF genes, the quantitative real-time PCR was performed. To this end, the total RNA was extracted from ASCs according to the kit protocol (Sinaclon, Iran). cDNA was synthesized using 1μg of total RNA and a cDNA synthesis kit according to the manufacturer's instructions (Sinaclon, Iran). Real-time PCR was performed using specific primers (Tables 1 and 2) and SYBR green master mix (Sinaclon Iran) in Rotor-Gene™ 6000 sequence detection system (Corbett Life Science, Australia). The relative expression ratio of the target genes was calculated using standard curves and the $2^{-\Delta CT}$ (CT target gene - CT β-actin) formula. β-actin gene was also used as the reference gene.

Enzyme linked-immunosorbent assay (ELISA)

To evaluate the levels of IL-6 and TGF-β produced by the ASCs, the culture supernatant

Table 1. Designed primers sequences, product size, and annealing temperatures of the β-actin, COX-2, HGF, IDO, and PD-L1 genes used in a relative reverse transcription-polymerase chain reaction (RT-qPCR).

Genes	Primer sequence	Product size (bp)	Annealing temperature
β-actin	Forward: GCCTTTGCCGATCCGC Reverse: GCCGGAGCCGTTGTCG	90	62
Cox-2	Forward: CTCAGCCATACAGCAAATCC Reverse: TCCGGTGTTGAGCAGTTTTC	134	58
HGF	Forward: TTCCATTCACTTGCAAGGC Reverse: CGTCCTTTACCAATGATGC	163	55
IDO	Forward: ATGTCCGTAAGGTCTTGCC Reverse: GTCCATGTTCTCATAAGTCAGG	155	56
PD-L1	Forward: AAGTCAATGCCCCATACAAC Reverse: CAGGACTTGATGGTCACTG	140	58

Cyclooxygenase-2 (COX-2); Programmed death-ligand 1 (PD-L1); Hepatocyte growth factor (HGF); Indoleamine 2,3-dioxygenase (IDO)

Table 2. To evaluate the levels of IL-6 and TGF-β by PCR protocol for real-time PCR

PCR protocol	Time	Temperature
Initial denaturation	5 min	95
Cycle		
Denaturation	30 sec	95
Annealing	30 sec	Variable (Based on each primer)
Extension	30 sec	72
Repeat cycles	40	-
Final extension	5 min	72
Melting curve analysis	5 min	72-95

was collected from all the groups and stored at -70 °C for further ELISA assays. Commercial ELISA kits were used to evaluate both IL-6 (BioLegend, USA) and TGF-β (Mabtech, Inc, Sweden). All the procedures were done according to the kits' instructions and the concentrations were calculated according to the standards provided in the kits.

Statistical Analyses

SPSS V.16 software (IBM Analytic, USA) was used for all the statistical analyses. To find significant differences in terms of the proliferation results, genes expression, and cytokine levels, one-way ANOVA and Tukey's multiple comparison test was performed. *P<0.05, **P<0.01, and ***P<0.001 were considered significantly different.

RESULTS

Characterization of ASCs

The isolated cells from adipose tissue were spindle-shaped and could adhere to the plastic surface (Figure 1A). According to the flow cytometry results, these cells were positive for CD105, CD73 and CD90 cell surface markers, while they lacked CD45 and CD34 (Figure 1B). These cells were also capable of differentiation into osteogenic, adipogenic and chondrogenic lineages (Figure 1C).

Proliferation of PBMCs

The effect of LDN-treated ASCs on the proliferation rate of allogeneic PBMCs was investigated in two-way MLR/ASCs co-culture using the colorimetric XTT method and the results were reported as the means of normalized proliferation ratios. The mean proliferation ratios of the control, 10^{-8}, 10^{-7} and 10^{-6} M NTX groups on day 2 of co-culture were (0.34±0.02), (0.41±0.03), (0.32±0.05) and (0.36±0.03), respectively. No significant difference was observed between the proliferation ratios of subjected groups compared with the control on day 2 ($P>0.05$). The mean proliferation ratios on day 4 of the experiment in the control, 10^{-8}, 10^{-7} and 10^{-6} M NTX groups were (0.91±0.01), (0.79±0.001), (0.88±0.06) and (0.89±0.05), respectively ($P>0.05$, Figure 2).

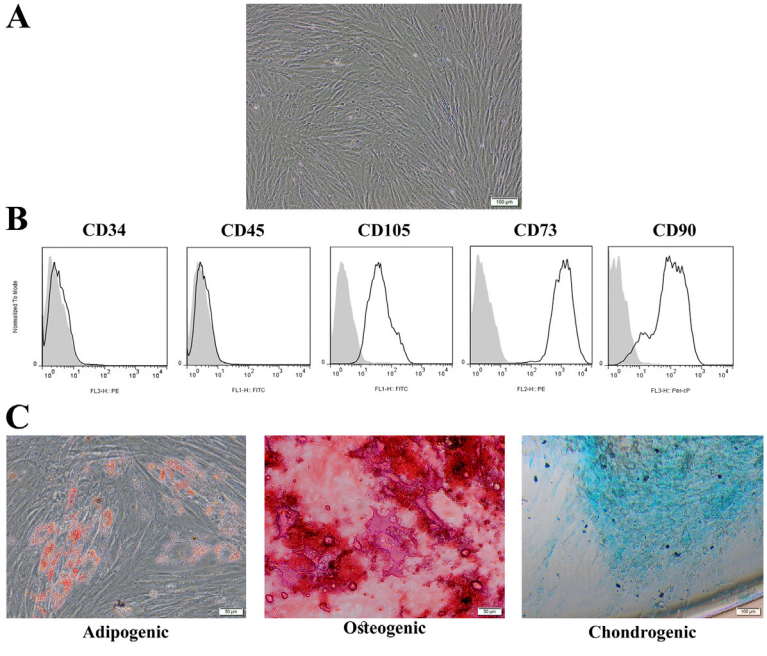

Figure 1. Characterization of ASCs. (A) The light microscopic morphology of the ASCs at passage 4. (B) The flow cytometry profile of the ASCs. ASCs in passage 4 were positive for the surface markers of CD 105, CD 90, and CD 73, while they were negative for CD 34 and CD 45. (C) The light microscopic illustration of adipogenic, osteogenic, and chondrogenic differentiation of the ASCs, featuring fat vacuoles, calcium deposits, and proteoglycan formation respectively. ASCs: Adipose Stem Cells; MSC: Mesenchymal stem cells

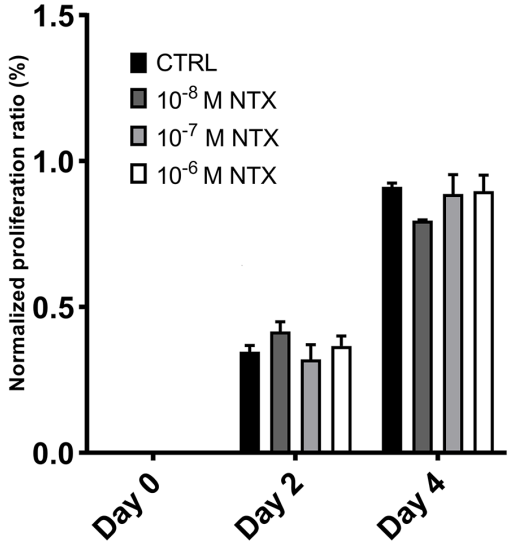

Figure 2. Effect of ASCs pretreated with different concentrations of LDN on the proliferation rate of allogenic PBMCs in two-way mixed leukocyte reaction (MLR)/ASCs co-culture. PBMCs proliferation rate was evaluated on Days 0, 2, and 4 of the co-cultures using XTT and reported as normalized proliferation ratio. No significant difference was observed between proliferation ratios of subjected groups compared to control on Day 2 and 4 (P>0.05). Normalized proliferation ratio=1-(O.D. of relevant Day/O.D. of Day 0). LDN: Low dose naltrexone; ASCs: Adipose stem cells; PBMCs: Peripheral blood mononuclear cells; NTX: Naltrexone

Gene Expression

Relative expression of COX-2, PD-L1, IDO, and HGF genes in ASCs was examined by real-time PCR, and the results were reported as relative expression to reference gene based on $2^{-\Delta CT}$ formula. The expression of these genes in stimulated and unstimulated conditions is reported below. In unstimulated condition, the mean expression ratios for the COX-2 gene in the control, 10^{-8},10^{-7} and 10^{-6} M NTX groups were (0.04±0.02), (0.1±0.05), (0.074±0.06) and (0.044±0.019), respectively (P>0.05). The mean expression ratios of PD-L1 in the control, $10^{-8,}$ 10^{-7} and 10^{-6} M NTX groups were (0.0004±0.0001), (0.0003±0.0001), (0.0004±0.0001) and (0.0005±0.00003), respectively (P>0.05). The mean expression ratios for the IDO gene in the control, $10^{-8,}$ 10^{-7}, and 10^{-6} M NTX groups were (0.00004±0.00005), (0.00002±0.00001), (0.000006±0.000009) and (0.0001±0.00005), respectively (P>0.05). The mean expression ratios of the HGF gene in the control, $10^{-8,}$ 10^{-7} and 10^{-6} M NTX groups were (0.013±0.003), (0.014±0.001), (0.015±0.003) and (0.02±0.004), respectively (P>0.05).

In stimulated condition, the mean expression ratios of COX-2 in the control, 10^{-8}, 10^{-7} and 10^{-6} M NTX groups were (0.077±0.003), (0.1±0.03), (0.06±0.01) and (0.05±0.01), respectively (P>0.05). PD-L1 gene mean expression ratios in the control, 10^{-8} and 10^{-7} M NTX groups were (0.002±0.0007), (0.003±0.001), and (0.002±0.0002), respectively (P>0.05). The expression of this gene in the 10^{-6} M NTX group was (0.005±0.001) and was significantly higher than in the control group (P=0.001). The mean expression ratios of IDO in the control, 10^{-8} and 10^{-7} M NTX groups were (0.15±0.02), (0.06±0.03) and (0.17±0.05), respectively (P>0.05). However, the mean expression ratio of the 10^{-6} M NTX group was (0.45±0.1), which was significantly higher than the rest of the study groups (P<0.0001). Also, the expression of this gene in the 10^{-7} M NTX group was higher than the 10^{-8} M NTX (P<0.05). For the HGF gene, the mean expression ratios of the control, 10^{-8}, 10^{-7} and 10^{-6} M NTX groups were (0.006±0.002), (0.006±0.001), (0.008±0.002) and (0.009±0.0006), respectively (P>0.05) (Figure 3).

Cytokine Level

IL-6 and TGF-β levels were assessed by ELISA and reported as pg/ml. The concentration of these cytokines in stimulated and unstimulated conditions is reported separately below.

In unstimulated condition, the mean of IL-6 levels in the control, 10^{-8}, 10^{-7}, and 10^{-6} M NTX groups were (5.38±2.13), (638.5±170.51), (1542.25±406.32) and (1746.25±617.94) respectively (P>0.05). The mean of TGF-β levels in the control, 10^{-8}, 10^{-7}, and 10^{-6} M NTX groups were (9.4±0.71), (1569.65±91.72), (1647.75± 110.44) and (743.15±173.01), respectively.

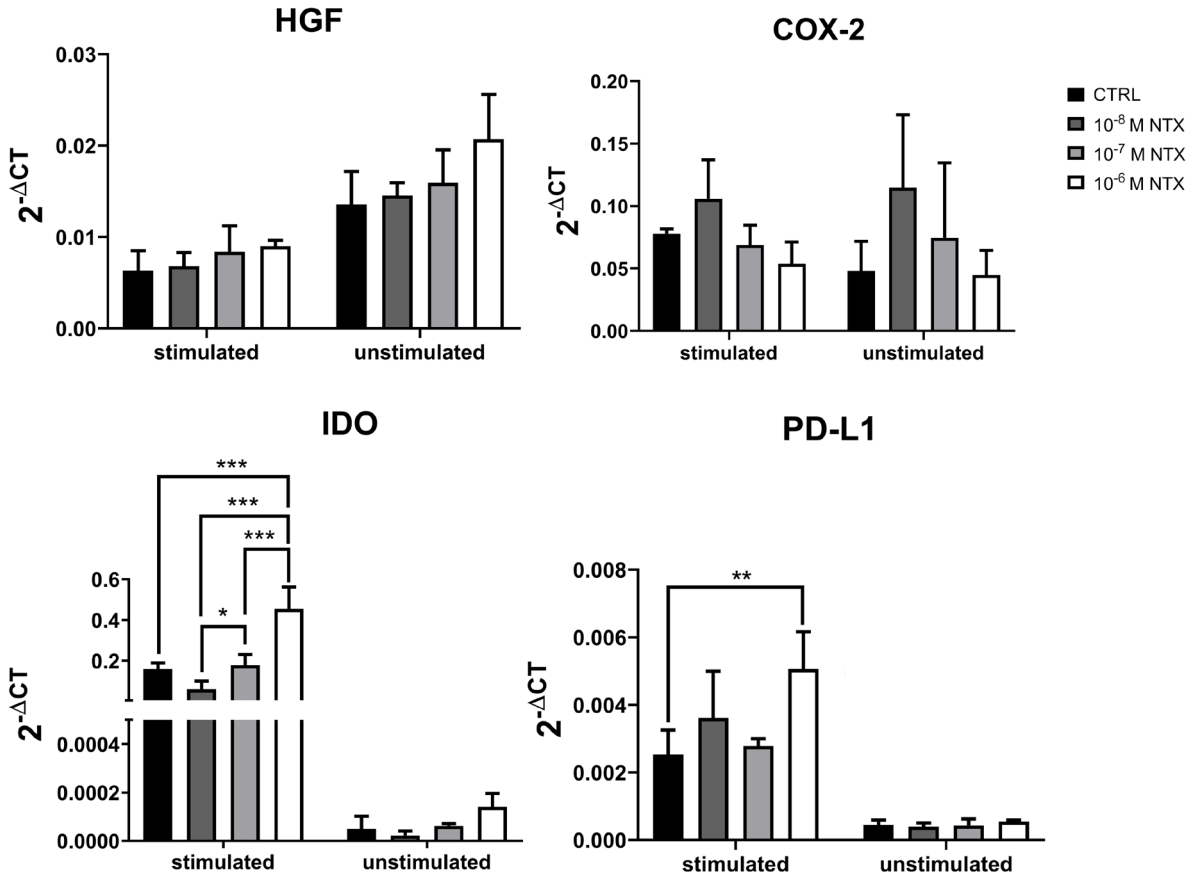

Figure 3. Relative expression of COX-2, HGF, IDO, and PD-L1 genes in ASCs. Gene expression was evaluated by real-time RT-PCR and the expression ratio is shown as a mean of $2^{-\Delta CT}$. There was no significant difference in the COX-2 and HGF expression between LDN treated ASCs and their control in both stimulated and unstimulated conditions (P>0.05). In the stimulated condition, the expression ratio of IDO in the 10^{-6} M NTX group was significantly higher than control, 10^{-8} and 10^{-7} M NTX groups (P<0.001). Moreover, IDO expression in the 10^{-7} M NTX group was higher than the 10^{-8} M NTX group (P<0.05). In this condition, PD-L1 had a higher expression in the 10^{-6} M NTX group than the control (P<0.01). *** indicates a significance level of below 0.001, ** indicates a significance level of below 0.01 and * indicates a significance level of below 0.05. ΔCT=(CT target gene-CT β-actin). COX-2: Cyclooxygenase-2; HGF: Hepatocyte growth factor; IDO: Indoleamine 2,3-dioxygenase; PD-L1: Programmed death-ligand 1; ASCs: Adipose stem cells; LDN: Low dose naltrexone

The level of this cytokine in $10^{-8,}$ $10^{-7,}$ and 10^{-6} M NTX groups were higher than in the control, for these comparisons, significance levels were (P<0.0001), (P<0.0001) and (P=0.001), respectively. Also, the TGF-β level in 10^{-6} M NTX group was significantly lower than in 10^{-7}, and 10^{-8} M NTX groups, (P<0.0001) and (P<0.0001), respectively.

In stimulated condition, the mean of IL-6 levels in the control, 10^{-8}, and 10^{-7} M NTX groups were (2786.25±526.64), (24212.5±4180.79), (23710.75±816.81) and (24328.75±2739.033), respectively. IL-6 level in all the three treated groups was significantly higher than that in the control group (P<0.0001). The mean level of TGF-β in the control, 10^{-8}, 10^{-7}, and 10^{-6} M NTX groups were (830.8±68.21), (431.6±59.03), (819.4±359.28), and (511.75±20.28), respectively (P>0.05, Figure 4)

To elucidate the relationship between IL-6 and TGF-β levels, we assessed IL-6/TGF-β ratios. IL-6/TGF-β ratios in the unstimulated conditions in the control, 10^{-8}, 10^{-7}, and 10^{-6} M NTX groups were (0.43±0.38), (0.28±0.2), (0.77±0.52) and (1.29±1.80), respectively. IL-6/TGF-β ratios in the 10^{-6} M NTX group were significantly higher than in the control (P<0.05).

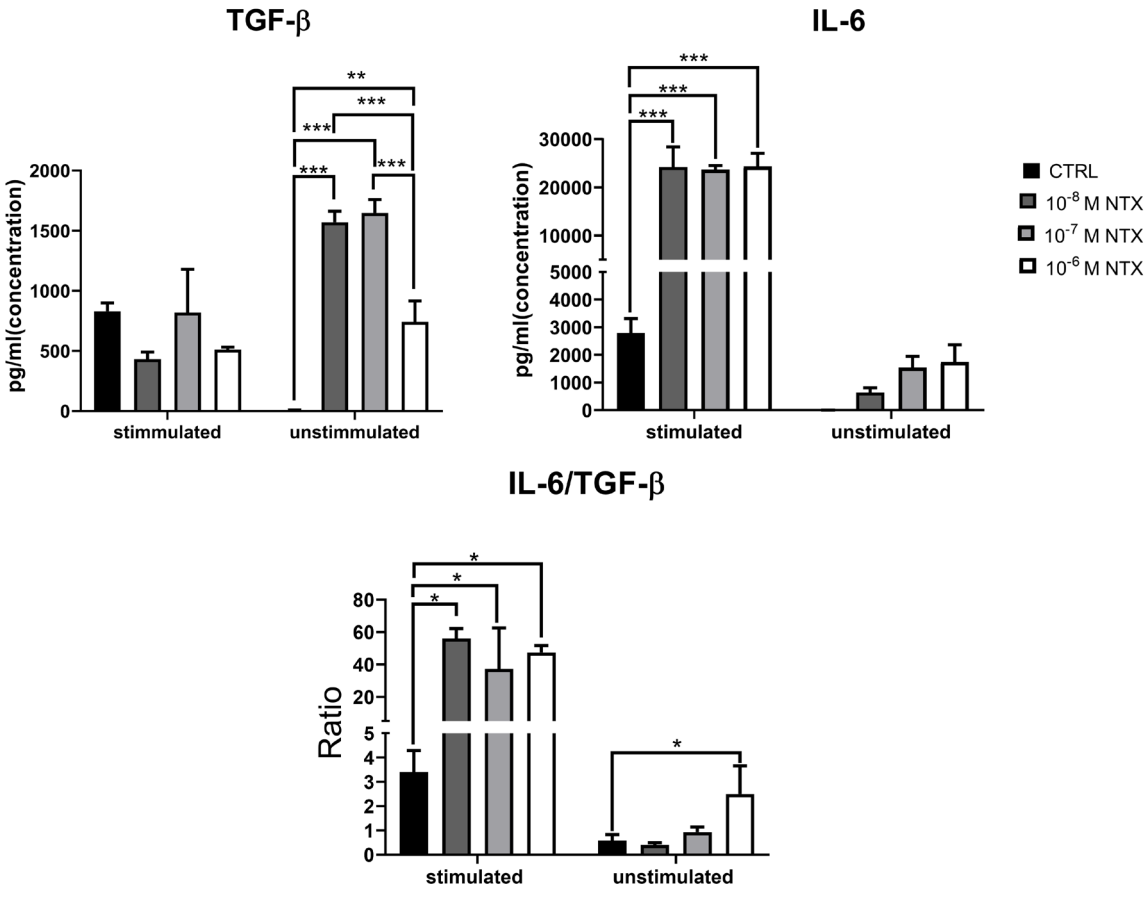

Figure 4. IL-6 and TGF-β levels in LDN treated ASCs. These cytokines level was measured by ELISA and reported via pg/ml. LDN treatment significantly increased IL-6 cytokine level in stimulated condition compared to control (P<0.001). The level of TGF-β in 10^{-8}, 10^{-7} and 10^{-6} M NTX groups in unstimulated condition were higher than control (P<0.001), (P<0.001), and (p<0.01). In this condition, the TGF-β level in the 10^{-6} M NTX group was lower than 10^{-8} and 10^{-7} M NTX groups (P<0.001). IL-6/TGF-β ratios in all LDN-treated groups were significantly higher relative to control (P<0.05). *** indicates a significance level of below 0.001, ** indicates a significance level of below 0.01, and * indicates a significance level of below 0.05. LDN: Low dose naltrexone; ASCs: Adipose stem cells; TGF-β: Transforming growth factor-β

IL-6/TGF-β ratios in stimulated condition in the control, 10^{-8}, 10^{-7}, and 10^{-6} M NTX groups were (3.4±0.88), (56.08±6.09), (37.16±25.4) and (36.50±24.59) respectively. IL-6/TGF-β ratios in all LDN-treated groups were significantly higher compared with the control (P<0.05, Figure 4).

DISCUSSION

Naltrexone hydrochloride, an antagonist of opioid receptors, is a medication for the treatment of physical and psychological dependence on opioids. This drug is also used in the management and treatment of inflammatory and various autoimmune diseases (6). Opioids, of which morphine is the best known, are generally referred to as suppressors of the immune system (18). Nevertheless, the immunomodulatory properties of morphine are dose-dependent and can exert various effects on different types of immune cells depending on the dosage (19). The treatment of the rat peritoneal macrophages with micromolar doses of morphine is reported to decrease NF-κB and pro-inflammatory cytokines, while nanomolar concentrations of morphine increase their levels (20). Furthermore, contrary to the analgesic effects of high morphine doses, lower doses of it can

increase sensitivity to pain (21). A similar difference can be observed in the behavior of opioid antagonists. Studies indicated the antagonistic effect of naltrexone at normal doses (50–100 mg), while low doses (0.001–1 mg) may behave similarly to opioids, such as morphine (22, 23).

Given that previous studies were mainly concerned with the LDN effect on different cells of the immune system, here, we examined the immunomodulatory properties of ASCs after 14 days of treatment with different LDN concentrations for the first time. In this research, we used the ASCs co-culture with a two-way MLR assay to investigate the effect of LDN-treated ASCs on the proliferation rate of allogeneic PBMCs. The expression of genes and the secretion of cytokines associated with the immunomodulatory function of treated ASCs were also assessed in unstimulated and IFN-γ stimulated conditions.

It was previously shown that MSCs are capable of reducing the proliferation rate of allogeneic PBMCs in two-way MLR/MSCs co-culture, also observed in our study (data not shown) (24). Our data did not show any significant changes in co-cultured PBMC proliferation under any concentrations of LDN compared with the control group. Although the LDN-treated ASCs did not elevate suppression of PBMC proliferation in comparison with the untreated ASCs, it should be noted that ASCs' ability to suppress PBMCs' proliferative response did not interfere with the LDN treatment. One of the possible explanations for these results could be the ASCs/PBMCs ratio used in our study design. Multiple studies demonstrated that different MSCs/PBMCs ratios can make remarkable differences in MSCs' immunomodulatory function in the MLR milieu (25). Also, it was suggested that cell-cell contact is the major mechanism of MSCs in suppressing PBMCs in the MLR model (26, 27). Our data shows that the LDN was more prone to alter the level of soluble factors of unstimulated ASCs like IL-6 and TGF-β. We think that the above-mentioned concerns can be responsible for the incapability of LDN-treated ASCs in altering the proliferation ratio of PBMCs in our study.

COX-2 is a molecule derived from arachidonic acid involved in the production of Prostaglandin E2 (PGE2). DONG Li et al. showed that umbilical cord MSCs transfected with the COX-2 gene pronounce more immunomodulatory properties than the MSCs transfected with other genes, such as IDO (28, 29). The role of COX-2 in reducing the function of T lymphocytes and other immune cells in the tumor microenvironment was previously demonstrated (30). Intrinsic expression of COX-2 under unstimulated conditions was reported in BM-MSCs, also observed in our study (31). According to research by Holan et al., the treatment of BM-MSCs with morphine increases COX-2 expression (15). Although a previous study stated a similar anti-inflammatory behavior of LDN to opioids, here in this study, the LDN treatment did not exhibit any significant changes in LDN-treated ASCs.

HGF is involved in cell regeneration, protection, and immunomodulation. HGF secretion by MSCs leads to the inhibition of DC and T lymphocyte activity (32). This molecule also binds to monocytes to produce IL-10 and promotes the differentiation of TH2 (33). In previous studies, intrinsic expression of HGF in MSCs was reported, seen in our results as well (34). Although other research has shown an increment of HGF in the stimulated conditions (35), an inverse result in the expression of this molecule was observed in stimulated ASCs of our study.

IDO is an intracellular enzyme that plays an essential role in the immunomodulatory function of MSCs, including a reduced proliferation of T lymphocytes (36). PD-L1 is also another important molecule expressed on MSCs and it is involved in the inhibition of T lymphocytes (28). Our findings indicated the effect of LDN treatment on the increment of IDO and PD-L1 gene expression in the stimulated ASCs. We also showed that ASCs treated with a higher concentration of LDN had more expression of IDO and

PD-L1 than their controls in stimulated condition. Previous studies also revealed that the expression of these genes in BM-MSCs significantly increased under the influence of IFN-γ (28, 37). In our study, not only was this effect of IFN-γ observed, but also LDN treatment significantly elevated IDO and PD-L1 gene expression. It was reported that morphine-treated BM-MSCs show higher expression of these two genes in the presence of pro-inflammatory cytokines (15). Therefore, increasing expression of IDO and PD-L1 genes in our study may be due to behavioral similarity of low concentrations of opioid antagonists to opioids themselves.

IL-6 is a cytokine secreted by MSCs and involved in the stimulating and differentiation of DCs as well as B and T lymphocytes (38, 39). Stimulation of BM-MSCs with IFN-γ significantly increases IL-6 expression, and a similar effect was seen in ASCs of our study (40). Various results were reported regarding the effect of LDN on IL-6 in previous investigations. For example, in the study by Parkitney, LDN reduces various pro-inflammatory cytokines (e.g. IL-6) in the sera of patients with fibromyalgia and decreases symptoms severity (14). However, in another study, LDN treatment increased the expression of IL-6 level in a mouse model of EAE (41). In the present research, IL-6 secretion increased in both stimulated and unstimulated conditions. Moreover, we observed greater alterations of IL-6 levels in stimulated condition relative to unstimulated conditions. Regardless of the concentration, LDN treatment significantly increased IL-6 in stimulated condition, but there was no clear pattern between LDN concentration and alteration trend of IL-6. Elevated IL-6 level might be the outcome of the suggested agonistic effect of LDN on opioid receptors (42).

On the other hand, we believe that the enhancement of IDO and PD-L1 gene expression in LDN-treated ASCs of the stimulated condition might be due to the interaction between IL-6 and the above-mentioned genes. Previous studies have shown that IL-6 via the JAK/STAT signaling pathway induces the expression of IDO in the Neuro 2a cell line (43), in which these two mediators are continually strengthening each other by establishing a positive feedback loop (44, 45). Also, Zhang et al. showed that the high level of IL-6 in serum activates the signaling path of stat/c-myc, increasing the expression of PD-L1 in peripheral blood monocytes which resulted in the induction of exhausted T cells (46). The results of Li et al. showed that TNF-α and IL-6 secreted by adipocytes increase the PD-L1 expression in HepG2 and B16F1 cells (47). Another study showed that in viral infections of the central nervous system, secreted IL-6 along with IFN-γ led to the increment of PD-1 and PD-L1 expression, consequently causing a delay in $CD8^+$ T-cell function (48). IL-6 is known as a pleiotropic agent, yet, recent studies show IL-6 induces the production and accumulation of myeloid-derived suppressor cells (MDSCs) in an inflammatory condition which leads to immunosuppressive phenotype in immune cells (49). However, despite the well-known inflammatory effects of IL-6, the above-mentioned studies indicate the immunosuppressive potential of this cytokine and brought us the idea that our study's IL-6 increment in LDN-treated ASCs of the stimulated condition might be responsible for the enhanced expression of anti-inflammatory mediators such as IDO and PD-L1.

TGF-β plays a role in various processes such as homeostasis, regeneration, evolution, and immunomodulation. This molecule is secreted by MSCs and affects the activity of immune system cells such as NK cells, T lymphocytes, and macrophages. According to previous studies, TGF-β expression increased in BM-MSCs when exposed to inflammatory cytokines like IFN-γ, also observed in our ASCs (50). Although TGF-β is mainly recognized as an anti-inflammatory cytokine, it is involved in the differentiation and activation of TH17 and Dermatitis, which also proves its pro-inflammatory effect (51). According to our results, in the unstimulated

condition, treatment with LDN significantly increased the production of this cytokine in ASCs. IFN-γ stimulation increased TGF-β in the control group, while LDN treatment did not alter the level of this cytokine in stimulated groups. In the Parkitney study, the injection of LDN for 8 weeks to patients suffering from fibromyalgia reduces the TGF-β level (14). Based on Walia et al.'s research, the treatment with TGF-β decreases IL-6-stimulated intercellular adhesion molecule-1 (ICAM-1) expression in an intestinal epithelial cell line (52). Kouichi Ohta et al. showed that IL-6 by suppressing TGF-β production in a mouse's aqueous humor terminates the immune privilege in the eyes of the Uveitis model (53).

In our study, we assessed the ratio of IL-6/TGF-β. In an unstimulated condition, IL-6/TGF-β ratios were below 1 in the control, and 10^{-8}, 10^{-7} M NTX, indicating the upper hand of TGF-β in the above-mentioned groups, while the treatment with 10^{-6} M NTX changed this balance towards IL-6. In the stimulated conditions, LDN treatment dramatically elevated the IL-6/TGF-β ratio in every group of this condition compared with their control. Our findings here could indicate the profound impact of LDN on the secretion of IL-6 and brought us this notion that the observed changes in TGF-β levels are not directly affected by LDN treatment and are probably induced in response to alteration of other LDN-related agents, including IL-6.

CONCLUSION

The current study is one of the first studies providing evidence of LDN effects on ASCs. Considering the diversity of LDN effects on immune cells and responses and given the importance of MSCs' immunomodulatory function and their role in shaping immune responses, here we aimed to investigate the effect of the prolonged LDN treatment on ASCs. Overall, we found that LDN induces a variety of different pro and anti-inflammatory aspects of ASCs function. The most considerable effect of LDN was observed in increasing the levels of secreted IL-6 from ASCs. Although this result appears to be in favor of immune response, we also witnessed an increment in the expression of immunomodulatory-related genes of PD-L1 and IDO. Therefore, based on the pivotal role of IL-6, we speculate the increment in these genes was due to the rise of IL-6 and its profound effect on them. It is also noteworthy that the above-mentioned effects were observed more clearly in higher concentrations of LDN. This finding suggests a similar effect between LDN and morphine and helps with the previous claims indicating the agonistic effects of these two molecules. We understand that our data cannot provide sufficient evidence for the suggested hypotheses, but it also provides the first insight into the LDN effects on ASCs. We believe that a better understanding of LDN effects on immune responses and MSCs' contribution in them requires further investigations.

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