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RESEARCH ARTICLE

# Mesenchymal stem cell -derived exosomes as natural nanoparticles (NPs) stimulate the growth of limbal stem cells (LSCs) by regulating Wnt/β-catenin, p38 MPAK and ERK pathway

***Amin Samiei [1], Parsa Aliabadi [2], Mohsen Parandavar [3], Mohammad Shirvani [4], Mohamad Saeid Hoseinzade Firozabdi [5], Navid Fazlinejad [6*]***

*[1] Oral and maxillofacial pathology department, Shiraz university of dental science, Shiraz, Fars, Iran*

*[2] Department of Immunology and Biology, Tehran University of Medical Sciences, Tehran, Iran*

*[3] Acute surgical department, Rajaee Hospital, Shiraz University of medical science. Shiraz, Iran.*

*[4] Poostchi Ophthalmology Research Center, Department of Ophthalmology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*

*[5] Department of Optometry, School of Rehabilitation Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

*[6] Department of Ophthalmology, Khodadoust Eye Hospital, Shiraz, Iran*

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**ABSTRACT**

**Objective(s):** In the current study, we investigated the impacts of the mesenchymal stem cell (MSCs)- exosome on limbal stem cells (LSCs) proliferation.

**Methods:** Exosomes firstly were isolated from the human MSCs. Then, they characterized by expression of CD9, CD63 and CD81using the western blotting and morphological evaluation by transmission electron microscopy (TEM) image. The proliferation levels of treated LSCs were investigated following treatment with MSCs-exosome by MTT assay during the 1-4 days of exposure at 50-400 ng/ml concentrations. The expression levels of the beta-catenin, Wnt, p38 MAPK, and ERK were measured in LSCs within 12 and 96 hours of exposure.

**Results:** MTT assay consequences exhibited that exosomes at concentrations of 50-400 ng/ml could boost the proliferation of the LSCs in vitro. Also, treatment caused the up-regulation of the expression of beta-catenin, Wnt, p38 MAPK, and ERK, partially or efficiently, in the LSCs.

**Conclusions:** We suggested that the exosome is capable of provoking the proliferation of the LSCs by up-regulating the proliferation involved pathways.

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## INTRODUCTION

Stem cells (SCs) exhibit the self-renewal competences and the capability to establish the specific cell types as well as an entire organism [1, 2]. Adult stem cells (ASCs) are unique types of the human stem cells complicated in an internal restoration mechanism and producing replacement for cells degenerated by various procedures [3]. They exist in at particular anatomical sites and may continue to be quiescent for long periods till induced by their necessity to sustain tissue homeostasis. Meanwhile, SCs in the cornea tissue are found mainly at the corneoscleral limbus [4, 5]. Indeed, two chief kinds of ASCs are found in the limbal niche, encompassing the epithelial and stromal stem cells [(or termed limbal stem cells (LSCs)] accompanied with the corneal stromal stem cells (CSSCs) [6]. These SCs support the transparency of corneal. The LSCs, as a well-characterized population of ASCs, situated in the corneal limbus' basal epithelial layer, causing

* Corresponding Author Email: *nfn1983@gmail.com*

the corneal epithelium regeneration. The LSCs deficiency (LSCD) is considered as an a pathologic condition, which was specified by the dysfunction as well as degeneration of SCs precursors of the corneal epithelium. This condition is characterized by corneal conjunctivalization [7]. In ocular tissue, LSCs bring about a restoration basis of progenitor cells, eventually generating and replacing corneal epithelium. As a result, LSCs injury showed in LSCD could underlie stern visual disabilities [8, 9].

Current reports signified that pleiotropic influences of mesenchymal stem cells (MSCs) are largely stimulated by generating soluble paracrine ingredients, which include but not restricted to cytokines, growth factors, and genetic material [10-12]. Exosomes (30-100 nm in diameter) are the main sorts of nano-scale extracellular vesicles (EVs) secreted by stem cell, immune cells and etc [13-15]. In addition to the exosomes, apoptotic bodies (ApoB) and microvesicles (MVs) are other types of the EVs and are larger than 100 nm in diameter. Exosomes classically convey the fundamental cargos, in particular, miRNA, to the recipient cells. In fact, exosomes facilitate tissue recovery and favor orang function [16, 17]. Interestingly, exosomes exert similar effects to MSCs in new vessel formation, moderating immune responses, and inducing target cell proliferation [18].

In this study, we have evaluated the possible effects of the MSCs-derived exosome on the LSCs in vitro.

## METHODS

*Cell culture*

The human MSCs were purchased from the Royesh Stem Cell Biotechnology, Iran. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Capricorn Scientific, Germany) with 10% Fetal Bovine Serum (FBS) (Capricorn Scientific, Germany) and 1% pen/strep (Sigma-Aldrich, Germany).To obtain the LSCs, limbal rim derived from mouse eyes was dissected. Then tissue was cut rim into minor fragments, and break down the fragments with trypsinization. MSCs and LSCs were incubated at a 37°C humidified atmosphere, with CO2 5%.

*Exosome isolation*

The exosomes were procured from the conditioned media (CM) of the stem cell culture flask. Exosome isolation was conducted using the Total Exosome Isolation Reagent (Invitrogen). Conditioned media was centrifuged at 10000 × g for 30 min to eradicate the other EVs.

*TEM image*

The isolated exosomes' morphology was analyzed exploiting the transmission electron microscopy (FEI Tecnai 12, Philips, Netherlands).

*Western blotting*

Expression of the CD63 and CD81 was evaluated on the surface on cell and exosome by western blotting. In brief, cells were lysed using RIPA buffer and resultant lysate transported to the PVDF membranes (MDI Membrane Technologies, USA). Then, primary and secondary antibodies (Abcam, UK) were used to estimate the expression of CD63 and CD81proteins on exosome and cell surface.

*MTT assay*

The influences of the exosomes at increasing concentrations of 50 -400 ng/ml on the LSCs were investigated by MTT assay concerning MTT kit instructions ((Sigma-Aldrich, Germany)). The $0.5\times10^5$ cells were seeded firstly in each plate of the 96-well plates and kept for 24 hours. Then we added the 50-400 ng/ml exosome, and the cells were kept for 24-96 hours. Eventually, the 5 mg MTT/ml medium (10 µL) was added. Ultimately, the OD of wells was assessed at 570 nm wavelengths following about 4 hours of incubation.

*RNA extraction and cDNA synthesis and Quantitative PCR*

Using the RNX Plus solution kit (Sinaclon, Iran) total RNA isolation from LSCs was managed. Then the cDNA was generated by the high-capacity kit acquired from the Bioneer, USA. For estimation of the expression of ERK, p38 MAPK, Wnt and β-catenin at mRNA levels, quantitative PCR was accomplished. The synthesized cDNA, forward and reverse primers (Table 1), distilled water, and PrimeTime™ Master Mix acquired from the Idtdna, (U.S.A were employed.

*Statistical analysis*

Results from three independent tests showed in Mean ± SEM. Then Student T-test was managed in to define the statistical differences. Consequences were analyzed by SPSS.

Table 1 Real-time PCR primers

| Gene | | Primer (5′-3′) |
|---|---|---|
| **ERK** | F | CTACACGCAGCTGCAGTACATC |
| | R | GTGCGCTGACAGTAGGTTTGA |
| **P38 MAPK** | F | CGAAATGACCGGCTACGTGG |
| | R | CACTTCATCGTAGGTCAGGC |
| **B-catenin** | F | CAAGAGTGGATGAGTGTGT |
| | R | AGTAGCTGGCTGAACGAG |
| **Wnt** | F | GGGTATTGACCCATGTGGC |
| | R | ATTGCCCGAAATGCGAT |
| **β-actin** | F | CACCCGCGAGTACAACCTTC |
| | R | CCCATACCCACCATCACACC |

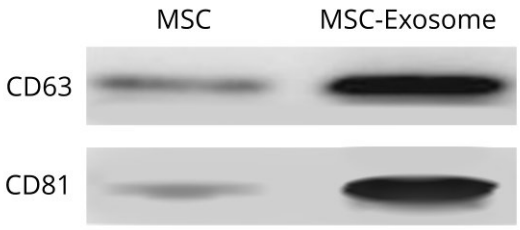


Fig. 1. The characterizing exosome using western blotting (CD63 and 81).

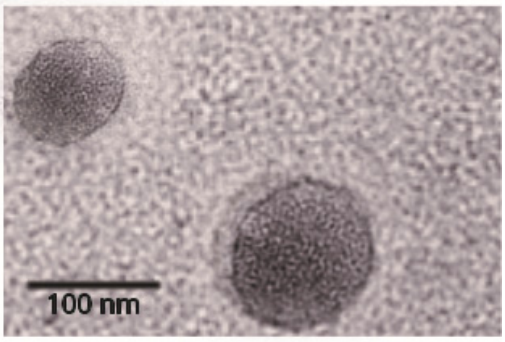


Fig. 2. TEM image of MSCs derived exosome in vitro

## RESULTS / DISCUSSION

*Western blotting and TEM image*

The expression of CD63 and CD81 and TEM image showed exosome successful isolation from MSCs (Fig. 1 and 2).

*MSCs-derived exosome boosted LSCs cell proliferation*

According to the MTT assay consequences, 50- 400 ng/ml concentrations of exosomes boosted LSCs proliferation within 24- 96 hours of intervention (Fig. 3). This influence was more prominent at the 400ng/ml concentration of exocome within 96 hours of treatment (Fig. 3). Indeed, the impact was both time- and dose-dependent. The supportive effects of the exosomes on LSC proliferation were significant but not strong at 50 ng/ml concentration.

The therapeutic competencies of MSCs to ameliorate corneal opacity, neovascularization, and deregulated immune response have strongly been evidenced in various experimental models of LSCD. In addition, it was exhibited that exosome from human MSCs could protect retinal ganglion cells (RGCs) and sustain retinal function in vivo [19].

*MSCs-derived exosome promoted p38 MAPK expression in LSCs*

The RT- PCR test was accomplished to assess p38 MAPK expression in LSCs following treatment with exosomes 50-400 ng/ml at 1 day and 4 day of exposure. Within 1 day of treatment, exosomes 50, 100 and 200 ng/ml did not affect p38 MAPK expression in LSCs (P<0.05) (Fig. 4). Nonetheless, exosomes 100, 200 and 400 ng/ml led to an increase in p38 MAPK expression at 4 days of intervention (P<0.05) (Fig. 4). Besides, 50 ng/ml concentration of exosome did not affect the expression of p38 MAPK at 4 days of treatment.

It previously recognized that induction of the p38 MAPK signaling may stimulate LSCs proliferation [20]. Thus up-regulation of this protein as shown upon exposure with exosome may be implicated in LSCs proliferation.

*MSCs-derived exosome promoted ERK expression in LSCs*

Based on the real-time PCR results, at 1 day of treatment, exosomes 100, 200 and 400 ng/ml but not 50 ng/ml improved ERK expression in LSCs (P<0.05) (Fig. 5). Besides, exosomes 50, 100, 200 and 400 ng/ml up-regulated ERK expression at 4 days of treatment (P<0.05) (Fig. 5).

Based on the previous studies, ERK pathway plays key role in regulating the proliferation of LSCs and other cell types of the ocular tissue [21-23]. Meanwhile, MSCs-exosome enhanced RGCs proliferation by activation PI3K/Akt and ERK pathway [24].

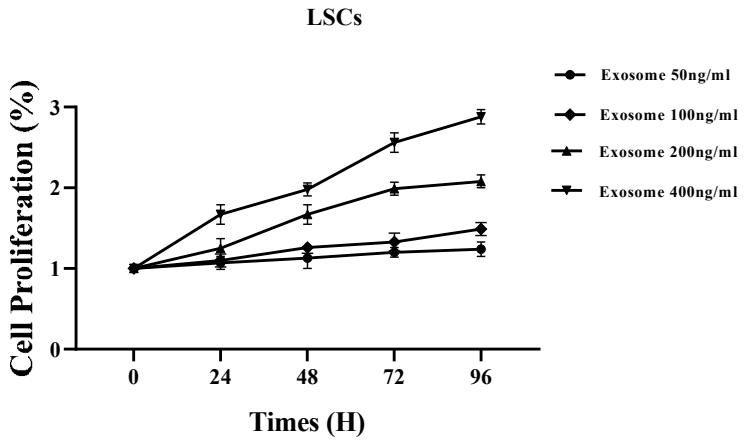


Fig. 3. The results of the MTT assay

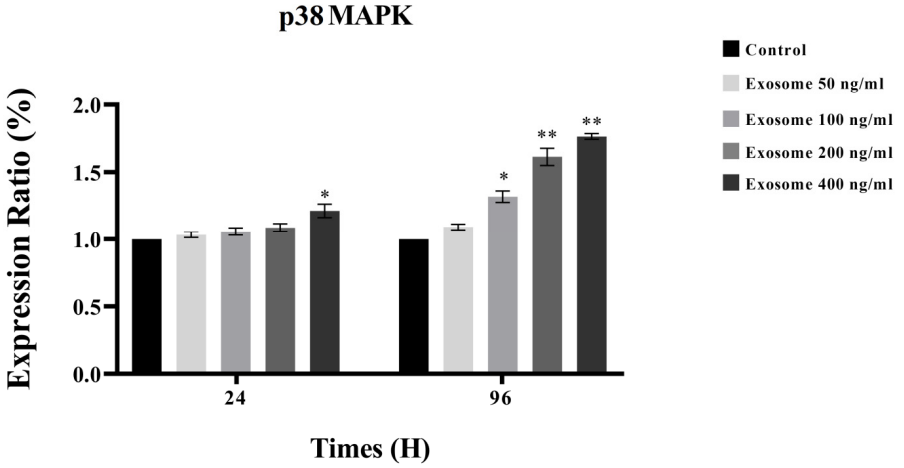


Fig. 4. The p38 MAPK expression in LSCs upon treatment with exosomes.

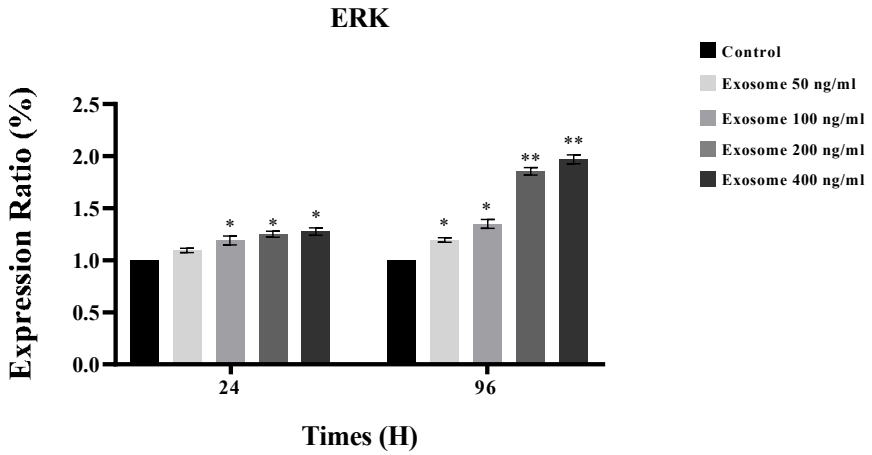


Fig. 5. The ERK expression in LSCs upon treatment with exosomes.

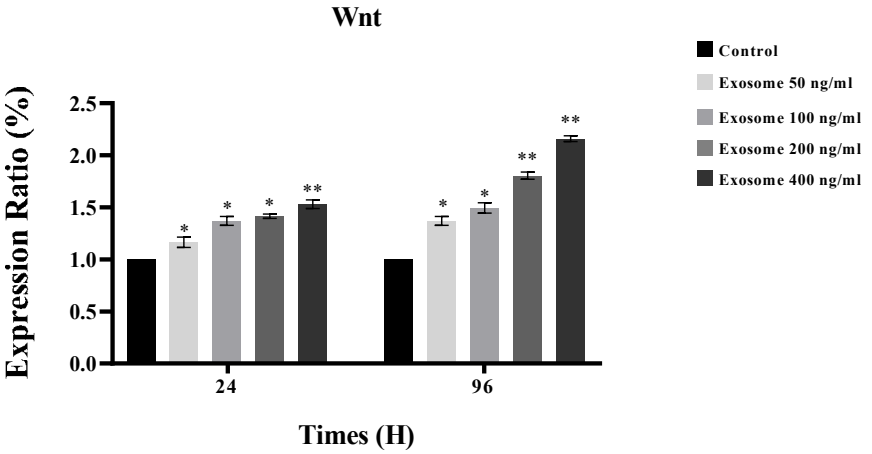


Fig. 6. The Wnt expression in LSCs upon treatment with exosomes.

*MSCs-derived exosome promoted Wnt expression in LSCs*

Based on the real-time PCR results, within 1 and 4 days of treatment, exosomes 50, 100, 200 and 400 ng/ml enhanced Wnt expression in LSCs ($P<0.05$) (Fig. 6). This effect was more evident within 96 hours of treatment and 400 ng/ml concentration ($P<0.05$) (Fig. 6).

*MSCs-derived exosome promoted β-catenin expression in LSCs*

The real-time PCR test was managed to estimate β-catenin expression in LSCs following treatment with exosomes 50-400 ng/ml at 1 day and 4 day of exposure. During 24 hours of treatment, only exosomes at 400 ng/ml concentration enhanced β-catenin expression in LSCs ($P<0.05$) (Fig. 7). However, during 96 hours of treatment, exosomes at 100, 200 400 ng/ml but not 50 ng/ml concentration up-regulated β-catenin expression in LSCs ($P<0.05$) (Fig. 7).

Theire is other evidence indicating that MSCs-derived exosome also can ameliorate liver fibrosis by inducing the Wnt/β-catenin pathway [25]. This pathways seems that one of the most central target of the human exosome.

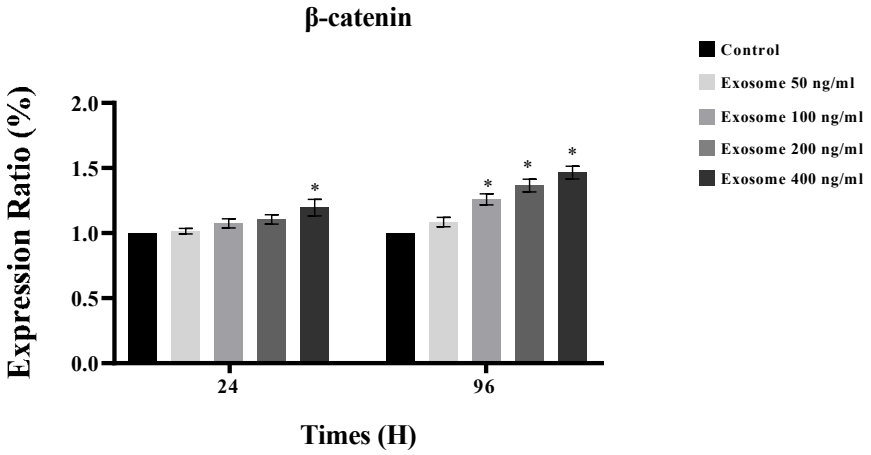


Fig. 7. The β-catenin expression in LSCs upon treatment with exosomes.

## CONCLUSION

Results exhibited that human MSCs-derived exosomes are capable of elicitingthe prolifertion of LSC by up-regulating the expression of ERK, Wnt, B-catenin, p38 MPAK. Theses finding higlithes the potential of exosome as a novel therapeutic modality to treat LSCD. This study like other increasing evidence suggested that paracrine mechanisms particpates in MSCs-induce effects in vivo, and exosomes are an key constituent of this paracrine activity.

## CONFLICT OF INTEREST

There is no conflict of interest.

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