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RESEARCH ARTICLE

Extracellular Vesicles isolated from mesenchymal stem cell (MSCs) negatively regulates human T cell survival by inducing caspase cascade and inhibiting anti-apoptotic proteins

Hamed Mahmoudi [1*] **, *Pooya Jafari Doudaran*** [2]

[1] *Resident of oral and Maxillofacial Surgery, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran*

[2] *Student Research Committee, Qom university of Medical Sciences, Qom, Iran*

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ABSTRACT

Objective(s): Extracellular Vesicles (EVs) transports active biomolecules between human many cells. As natural nanoparticles (NP), exosomes (Exo), plays important roles in the diagnosis and also treatment, of human disorders. Recently, it has been exhibited that mesenchymal stem cell (MSC)-derived Exo could regulate immune cells' biological processes. We evaluated the influence of the MSCs-derived Exo on T cell survival.

Methods: Exo were procured from the supernatant of bone marrow (BM)-MSCs. The structure and form of MSC-Exo were investigated using transmission electron microscopy (TEM). Exo markers, CD9 and CD63, were detected by Western blotting. In co-culture condition with MSC-derived Exo, the survivals of T cells were assessed by flow cytometry. Also, the expression levels of the Caspase-3, Caspase-8, Bcl-2 and Mcl-1 were measured by Real-time PCR.

Results: MSCs-derived Exo induced apoptosis in human T cells more strongly within 72 hours but not 24 hours of co-culture. Real-time PCR results also showed that Exo co-culture led to down-regulation of Mcl-1, and Bcl-2 while inducing Caspase 3 and 8 expressions at mRNA levels.

Conclusions: MSCs-derived Exo could induce apoptosis in T cells and lead to immunomodulation by triggering caspase cascade and also down-regulating anti-apoptotic protein levels.

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INTRODUCTION

Mesenchymal stem cell (MSC) therapy provides an encouraging therapeutic modality for treating autoimmune diseases, sepsis as well as transplant surgery [1, 2]. They are positive for MHC-I, CD90, CD105, and CD73, while lacking the CD45, CD34, CD14, or CD11b [3]. MSCs could be isolated from various sources, such as bone marrow (BM), adipose tissue (AT), umbilical cord (UC), skin, muscle, liver, etc. They could be differentiated into the various cells like connective tissue, bone, cartilage, and etc [4, 5]. MSCs mainly elicit strong immunosuppressive influences, largely achieved by targeting T cell proliferation, deterring the activation of B cell activation, influencing the DCs activation [6, 7]. However, recent studies have shown that the risk of tumor formation and cell aging might bypass their widespread utility in clinic.

Recent examinations indicated that the pleiotropic influence of MSCs is mainly due to the producing soluble paracrine factors ranging from cytokines to genetic material [8, 9]. Exosomes (Exo), nanoscale extracellular vesicles (EVs), largely involves in the MSCs-mediated paracrine effect. They are 30-150 nm in diameter and are categorized as one of the 3 main sub-populations of the EVs. They convey efficient cargos such as miRNA and mRNA, proteins, and

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

lipids resulting from stem cell to the neighboring cells. Exo implicate in intercellular interactions and facilitate healing of damaged and destructed tissues and organs [10, 11]. Other sub-populations consist of microvesicles (MVs) and apoptotic bodies (ApoB) (both larger than 100 nm) [12]. Exo, as natural nanoparticles (NPs), are shaped by budding as intraluminal vesicles (ILVs) from the late endosomes luminal space [13, 14]. Many reports have evidenced that the Exo play central role in MSCs-mediated therapeutic effect on many experimental models. Importantly, they provoke similar effects to MSCs, more importantly, improving tissue repair, enhancing angiogenesis, immunmodulation, as well as neuroprotection [15, 16]. Indeed, Exo convey biological information between human cells and adjust in the physiological and pathological circumstances [17]. They express tetraspanin and CD63, CD9, CD81, and CD82. They are integrin proteins situated on the surface of Exo and participate in cell adhesion. Besides, Rab GTPases, Annexins, and HSP70 and HSP90 are other Exo-related proteins contributed to the membrane fusion [18, 19]. In vivo, MSC-Exo can inhibit the potential of peripheral blood mononuclear cells (PBMCs) to secret inflammatory biomolecules. Aside that, Exo can promotes anti-inflammatory TGF-β and IL-10 from PBMCs, thus triggering the growth and immunomodulatory capability of Tregs [20]. Furthermore, MSC-Exo can diminish the neuroinflammation in neurological diseases animal models by up-regulating Tregs activation and promoting M2/M1 macrophage ration [21]. However, the underlying cellular and molecular mechanisms behind the MSC-induced immunosuppression have not been completely explained.

In the current study, we examine the potent effects of the MSCs-Exo on immune T cells survival in vitro and also study the effect of Exo on caspase cascade and expression of the anti-apoptotic proteins in these T cells.

MATERIAL AND METHODS

Cell culture

Bone marrow (BM)-MSCs were purchased from Royesh Stem Cell Biotechnology (Tehran, Iran). Cells were cultivated in DMEM with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany) and 1% pen/strep. BM-MSCs then were maintained at a 37°C humidified atmosphere with CO2 5%.

To procure T cell isolation, human blood samples were prepared from healthy human donor. Human PBMCs were separated using ficoll density gradient centrifugation. Finally, human T cells were procured using MACS with respect to the producer recommendations. Isolated T cells were cultured in RPMI-1640 media (Sigma-Aldrich, Germany) enriched with 10% FBS, 1% pen/strep accompanied with 2 µg/mL phytohemagglutinin (PHA).

Exo isolation

BM-MSCs derived Exo were procured from the serum-free conditioned media by a MagCapture™ Exosome Isolation Kit PS (FUJIFILM Wako). The serum-free conditioned media was centrifuged at 10000 × g for 40 min to remove other types of the EVs. Then, supernatants were filtered by 0.2 mm filter membranes and ultimately were concentrated.

Transmission electron microscopy (TEM)

To evaluate the morphology of isolated Exo, these exosomes were examined by transmission electron microscope.

Western blotting

To determine the expression of Exo markers, including CD9 and CD63, the BM-MSCs-derived Exo and parental MSCs were firstly lysed in RIPA buffer containg the protease inhibitor (Thermo Fisher Scientific, U.S.A). Following the running of the lysate proteins on 10% sodium dodecyl sulfate-polyacrylamide gels, they were transported to polyvinylidene difluoride (PVDF) membranes. Then, primary and secondary anti-bodies (Abcam, UK) were used to detect proteins CD9 and CD63. Ultimately, the detection phase was conducted exploiting the Pierce ECL Western Blotting Substrate (Millipore).

Fluorescence-activated cell sorting (FACS)

Firstly, BM-MSCs-derived Exo (50ng/ml) were added to the culture media including human T-cells. An Annexin V-based kit (Dako, Denmark) was applied to determine the apoptosis percentages of the T cells within 24 and 72 hours of co-culture. After that, 10 µL of propidium iodide (PI) and 10 µL of FITC-conjugated Annexin-V were added into T cells and BM-MSCs-derived Exo including wells. Finally, the apoptosis levels were measured by a FACSCalibur, and the results were examined by FlowJo software.

RNA extraction and cDNA synthesis

The RNX Plus solution kit (Sinaclon, Iran) was

Table 1. Candidate genes' primer pairs for Real-Time PCR

Gene	Primer (5′-3′)	
Caspase-3	F	TGAGCCATGGTGAAGAAGGA
	R	TCGGCCTCCACTGGTATTTT
Caspase-9	F	AACCCTAGAAAACCTTACCCC
	R	CATCACCAAATCCTCCAGAAC
Bcl-2	F	GGATTGTGGCCTTCTTTGAG
	R	CAGCCAGGAGAAATCAAACAG
Mcl-1	F	GTTGGTCGGGGAATCTGCTA
	R	AAATTAATGAATTCGGCCGG
GAPDH	F	GAGTCAACGGATTTGGTCGT
	R	TTGATTTTGGAGGGATCTCG

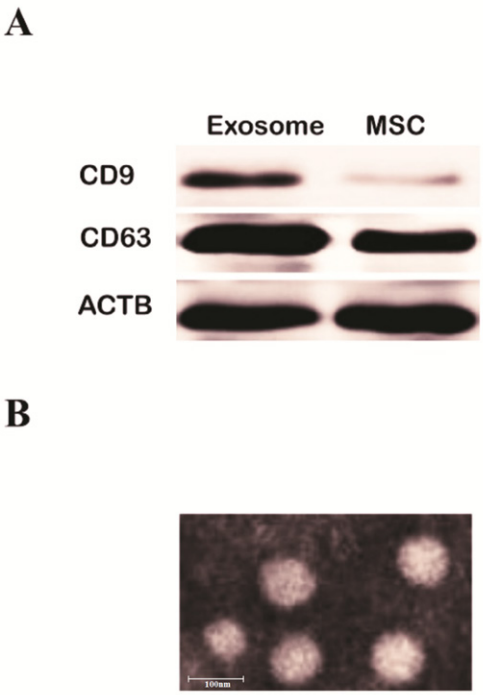

Fig. 1. Exo characterizing using western blotting (A) and also TEM image (B).

utilized to extract total RNA from T cells. Firstly, cells were lysed and the yields were mainatined at -70°C and thawed when RNA extraction was prerequisite. A high-capacity kit (Bioneer, USA) was exploited to establish complementary DNA (cDNA) from the yielded RNA.

Real-Time PCR

To examine the expression levels of Caspase-3, Caspase-8, Bcl-2 and Mcl-1 expression at mRNA levels, Real Time-PCR was conducted using cDNA, forward and reverse primers, distilled water, and PrimeTime™ Master Mix (Idtdna, USA) according to kit instructions. These genes expression ratio was assessed using Step One Plus Real-time PCR (Applied Biosystems, USA) in triplicate. This process was applied using pre-set cycling parameters. The GAPDH genes were employed to normalize the mRNAs expression. The primer pairs exploited in Real-Time PCR have been listed in Table 1.

Statistical analysis

Consequences are achieved from 3 or 6

independent experiments. Data are exposed in Mean ± SEM. Student T-test was conducted in order to determine the statistical differences. All analyses were performed by Graph Pad Prism software. Finally, the P value < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

The characterizing of Exo

The western blotting results validated the presence of CD63 and CD9 in BM-MSCs-derived Exo (Fig. 1A). Also, based on TEM images, the procured Exo showed a spherical shape (Fig. 1A).

MSCs-derived non-scale Exo induce T cell apoptosis

The apoptosis percentages of the human T cells were estimated in co-culture condition with Exo. The apoptosis percentage of T cells was measured at 24 as well as 72 hours of treatment with Exo using Annexin-V/PI staining and flow cytometry analysis. Consequences demonstrated that Exo did not induce apoptosis significantly in T cells within 24 hours of co-culture. Nonetheless, an enhancement in T cell apoptosis was found within 72 hours of co-culture ($P<0.05$) (Fig. 2A, B). The apoptosis levels in T cells (control) and T cells plus Exo within 24 hours of co-culture, and T cells plus Exo within 72 hours of co-culture were 4.78±1.41, 8.28±2.45, and 27.38±3.08, respectively (Figs. 2A, B).

Improved activity of Tregs modulates immune response and provide suitable environment to tissue regeneration. Other investigations also showed the inhibitory effects of the Exo derived from the adipose-MSCs on T cell [22]. Our findings indicate that MSCs-Exo could inhibit immune response by negative regulation of T cell survival and activity. Other studies also verified the positive effect of the MSCs-Exo on Tregs activity [23]. Meanwhile, Blazquez et al. (2014) found that Exo could diminish T cell proliferation and also bargained IFN-γ release [24]. Aside that, it has been proposed that BM-MSCs-Exo adjust B cells activation by targeting the expression of the miRNA-related genes [25]. These immunmodulatory properties make MSCs-Exo an authentically strategy to ameliorate immunological diseases [26].

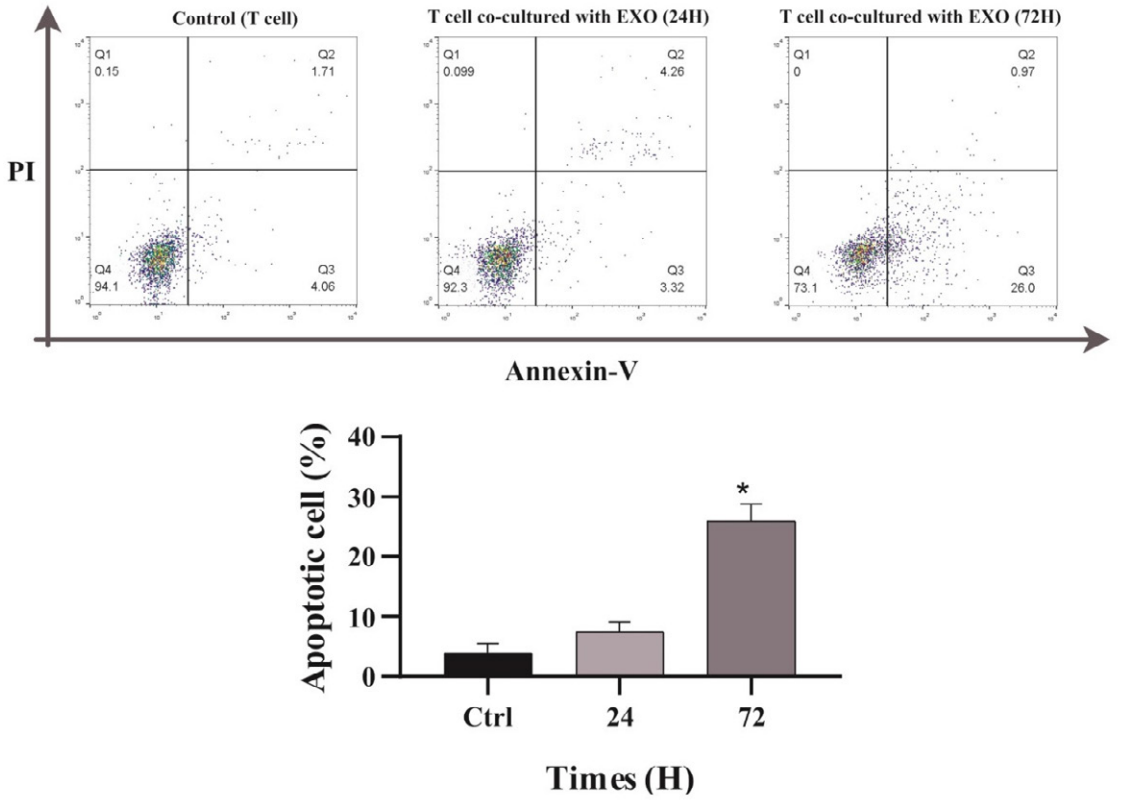

Fig. 2. Flow cytometry results of the effects of exosome on T cell apoptosis.

MSCs-derived Exo augmented Caspases expression in T cell

Real-time PCR test was accomplished to deterimne the expression levels of the Caspase-3 and Caspase-9 in T cell following co-culture with Exo during 24- 72 hours of treatment. Accordingly, Exo co-culture resulted in an increase in the Csspase-3 expression in T cells at 24- 72 hours of treatment ($P<0.05$) (Fig. 3A). Besides, Caspase-9 expression at mRNA levels was up-regulated at 48 and 72 hours but not 24 hours of co-culture with Exo ($P<0.05$) (Fig. 3B).

These Caspases play key roles in Caspase cascade activation [27, 28]. The up-regulation of the Caspases expression could be a putative mechanisms by which Exo exert its suppressive effects on human T cells. Caspase cascade activation eventually results in cleavage of a many proteins in the cell and facilities cell apoptosis [29]. Similarly, other reports exhibited that MSCs inhibited leukemia T cell proliferation by two main mechanismS: 1) up-regulation of the expression of Caspase-3 and -8 and 2) improving Bax/Bcl-2 ratio [30].

MSCs-derived Exo reduced the expression of anti-apoptotic protein levels in T cell

Real-time PCR test was managed to examine Mcl-1 and Bcl-2 expression in T cell upon co-culture with Exo within 24- 72 hours of exposure. According to analysis, Exo co-culture caused an attenuation in the Mcl-1 expression in T cells at 48 and 72 hours, while did not any effect at 24 hours of exposure ($P<0.05$) (Fig. 4A). Also, Bcl-2 expression at mRNA levels was reduced only within 72 hours but not 24 and 48 hours of exposure ($P<0.05$) (Fig. 4B).

Bcl-2 and Mcl-1 serve important roles in down-regulation of Caspase cascade activation [31]. They mainly overexpressed in leukemia and lymphoma and play imperative roles in their pathogenesis [32]. They inhibit cytochrome C release and consequently deter Caspase-9 activation [33].

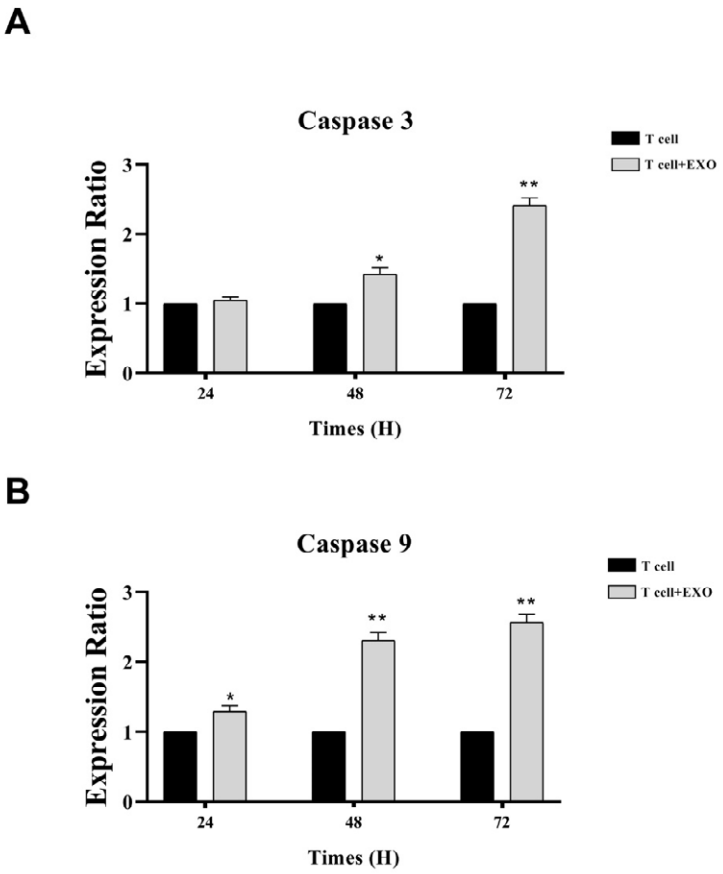

Fig. 3. Real-Time PCR information exhibiting Caspase-3 and Caspase-9 expression levels in T cells co-cultured with Exo within 24-72 hours of exposure.

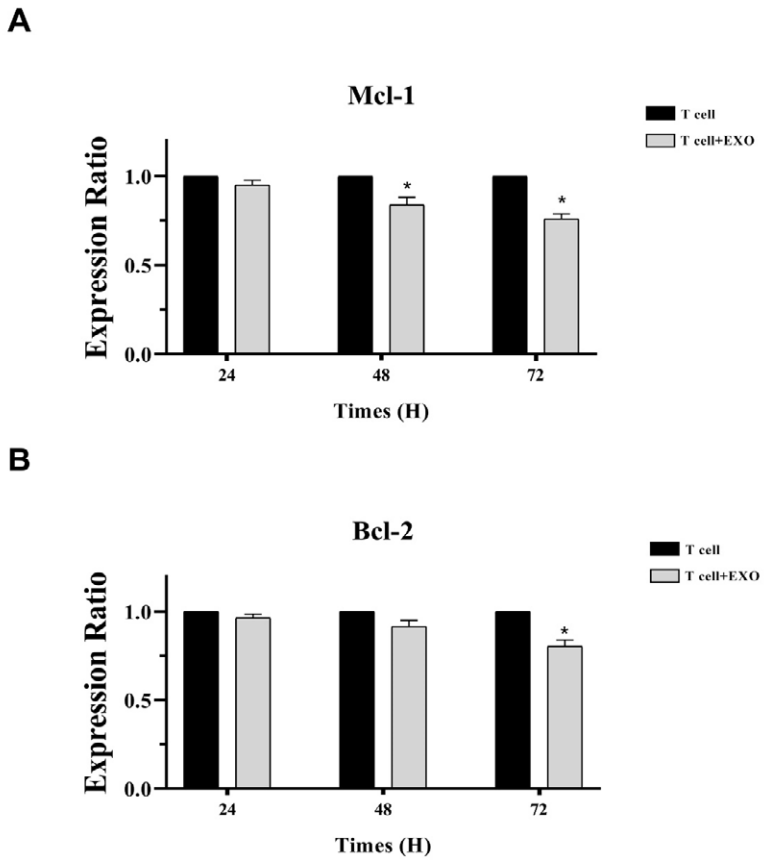

Fig. 4. Real-Time PCR results exhibiting Mcl-1 and Bcl-2 expression levels in T cells co-cultured with Exo within 24-72 hours of exposure.

CONCLUSION

It has beforehand been demonstrated that MSCs-Exo is capable of alleviating T cell viability, thus introdings Exo as a putative strategy to stimulate immunomodulation. In the present study, MSCs-Exo induced apoptosis in human T cells mainly by two mechanis: 1) inducing caspase expression and 2) down-regulating anti-apoptotic protein levels. The current challenge of this intervention is the low yield of exosomse and lack of the universally acceptable isolation methods. Also, the contamination with other vesicles has been observed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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