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ORIGINAL ARTICLE

Secretome of adipose derived-mesenchymal stem cells reduces the *Vibrio cholerae* attachment to Caco-2 cells and subsequent inflammatory responses

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ABSTRACT

Background and Objectives: Mesenchymal Stem Cells (MSCs) can repair gastrointestinal tract damage. The Secretome of MSCs has a high capacity to inhibit bacterial colonization and the subsequent inflammatory responses of *Vibrio cholerae.*
Materials and Methods: The Caco-2 cells were treated with adipose-derived MSCs (AD-MSCs) secretome and then infected with *V. cholerae.* Subsequently, the bacterial attachment and invasion, cholera toxin gene expression, PGE2 and IL-6 secretion, TNF-α, IL-1β, and IL-8 expression, and apoptosis of Caco-2 cells were evaluated.
Results: The secretome of AD-MSCs significantly reduced the *V. cholerae* attachment and internalization on Caco-2 epithelial cells (P<0.0001). The cholera toxin (Ctx-B) gene expression (FR=4.56 ± 0.66) and PGE2 production (P=0.0007) were also significantly reduced. The production of NO and TNF-α, IL-1β, and IL-8 pro-inflammatory cytokines were significantly (P<0.05) reduced in exposure to the secretome of AD-MSCs. Secretome also improved a significant 81.33% increase in IL-6 production (128.1 ± 37.6 pg/mL) and showed a 12.36% significant decrease in epithelial cell apoptosis (P< 0.0001) after exposure to *V. cholerae.*
Conclusion: The secretome of AD-MSCs can play a critical role in inhibiting bacterial colonization, and subsequent inflammatory responses, and maintaining the integrity of the epithelial barrier. The secretome may be effective in the prevention of hypovolemic shock.

Keywords: Inflammation; Apoptosis; Epithelium; Mesenchymal stem cell; *Vibrio cholerae*

INTRODUCTION

Vibrio cholerae is a Gram negative bacteria that is transmitted from infected food or water to the human population and causes acute dehydrating diarrheal disease. Until now, the world has undergone seven pandemics and Cholera is endemic in more than 50 countries that annually affects 2 to 3 million people in the world. The ability of the bacteria to intestinal mucus penetration, epithelium attachment and Cholera toxin production is necessary for disease formation. The world has undergone 7 pandemics and now Cholera is endemic in 50 districts, and it has an annual population of 2 to 3 million people in the world. Cholera toxin as the main virulent factor of *V. cholerae*, mediates enzymatic activation of adenylate cyclase and increases cAMP which terminates dehydration through diarrhea (1).

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After *V. cholerae* infection extensive neutrophil infiltration and mast cell/eosinophil degranulation appeared. Different inflammatory molecules including cytokines, chemokines, leukotriene B4, and many bactericidal factors like myeloperoxidases released at the infection site, provided the first innate immunity responses. Following the activation of innate immunity, B-cell mediated immune responses occur, and different antibodies from various isotypes are induced against *V. cholerae* LPS or other specific antigens such as cholera toxin. Although both systemic and mucosal antibody production increased rapidly after infection, they are not protective enough because reduced to baseline levels during a short time post infection (2).

Rehydration is the standard way of cholera management. However, there are some problems for optimal rehydration of patients with severe cholera at a critical time, especially in epidemics that indicate the necessity of adjunctive therapy. In the most cases of cholera infections, antibiotics are the first line of treatment in conjunction with hydration to reduce the duration and volume of diarrhea. However, the antibiotic resistance problem, its gastrointestinal side effects and providing adequate resources especially during outbreaks, prevents the complete success of this method (2).

With regard to what was mentioned above, finding new strategies to control infection induced damage and reducing mortality appears to be necessary. There is different evidence that inflammation aggravates cholera infection and its consequences. *V. cholerae* cytolysin (VCC) and OmpU virulent factors induce the production of pro-inflammatory cytokines that mediate the pathologic effect of augmented inflammation (3). Proinflammatory cytokines like IL-8, IL-1β, IL-6, and TNF-α increase diarrhea by induction of histamine and prostaglandin release from mucosal mast cells and disruption of epithelial tight junction permeability (4, 5).

Recent studies demonstrated that suppression of inflammatory cytokines could inhibit tight junction (TJ) disruption and increase permeability (6). Prostaglandin E_2 (PGE2) and some inflammatory cytokines induced cAMP production that resulted in cystic fibrosis transmembrane conductance regulator (CFTR) opening, dysfunction of ion channels and increases in intestinal secretion. Anti-inflammatory cytokines like IL-10 suppress cAMP production and reduce chloride secretion. In addition, non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac and ibuprofen inhibit cyclooxygenase that terminated to reduce prostaglandin synthesis and diarrhea formation (2). In this regard, anti-inflammatory compounds can be proposed as a combined treatment.

Recent studies support both the anti-microbial and the anti-inflammatory effects of MSCs making them a good candidate for the control of infectious and inflammatory diseases. MSCs secrete profound immunoregulatory factors that suppress the activation of immune cells. Much evidence confirms that stimulation of MSCs with inflammatory signals like microbial products enhances their anti-inflammatory function. In addition, MSCs can recruit to gut epithelia and support this barrier by helping to repair damaged parts and proliferation of epithelial cells (7). According to these findings, it seems that MSCs can be stimulated with intestinal microorganisms and repair gastrointestinal tract damage through anti-inflammatory function and epithelial regeneration.

In spite of the mentioned evidence, our previous study showed that MSCs in direct contact with epithelial cells, induce *V. cholerae* internalization to epithelial cells followed by an increase in inflammatory cytokine production and apoptosis. We related this result to the formation of the peri-cellular zone of matrix metalloproteinases (MMPs) by MSCs, because MMPs provide the context for the attachment and entry of bacteria into epithelial cells (8). Since MSCs secrete large amounts of tissue inhibitor matrix metalloproteinases (TIMMPs) that inactivate metalloproteases, the present study was designed to investigate the effect of MSCs secretion on the attachment of *V. cholerae* to epithelial cells and its inflammatory responses (9). To this purpose, the Caco-2 cell line was treated with Adipose derived-mesenchymal stem cells (AD-MSCs) secretome and then infected with *V. cholerae*. Then, the effect of this treatment was determined on bacterial attachment and invasion, cholera toxin gene expression, prostaglandin E_2 (PGE_2) and interleukin-6 secretion, TNF-α, IL-1β and, IL-8 expression and apoptosis of Caco-2 cells.

MATERIALS AND METHODS

Mesenchymal stem cell culture, secretome isolation, and characterization. MSCs isolated from human abdominal fat by enzymatic digestion method. Primary MSCs were cultured in a High Glucose-Dul-

becco`s Modified Eagle Medium (HG-DMEM) supplemented with 10% FBS (Gibco, USA). After reaching 80% confluence, the cell supernatant was replaced with a DMEM culture medium containing 5% FBS. The MSCs secretome was collected after 72 hours and stored in a -70°C freezer until use.

The purity of human abdominal fat-derived MSCs was evaluated by immunophenotyping of CD45, CD105, CD90, CD73, and Sca-1 cell surface markers. The differentiation power of MSCs to adipocyte and osteocyte were also evaluated respectively by Alizarin Red (AR) and OilRedO(ORO) assays (10).

***Vibrio cholerae*.** In this study, *V. cholerae* ATCC14035 was used. Bacteria were grown in Brain Heart Infusion (BHI) Broth at 37°C for 20 hours and stored at -80°C in Luria-Bertani (LB) broth containing 30% (v/v) glycerol.

Caco-2 cell culture and treatment protocol. Caco-2 cells were purchased from the Iranian Biological Resource Center (IBRC) and expanded in DMEM supplemented with 10% FBS. For experimental studies, Caco-2 cells were seeded at 3×10^5/ wells of 12 wells plate to reach 100% confluency immediately after attachment. Cells were incubated in a 5% CO_2 incubator at 37°C for 10 days to differentiate into polarized epithelial-like monolayers. The cells were divided into four experimental groups. Group I were Caco-2 cells that received no treatment. Group II, were Caco-2 cells that were infected with *V. cholerae* for 2 h. Group III, were Caco-2 cells treated with AD-MSCs secretome supplemented with 10% FBS for 48 h. Group IV, were Caco-2 cells pretreated with AD-MSCs derived secretome for 48 hours and then infected with *V. cholerae* for 2 h. Careful washing was then carried out to remove unbound bacteria (Fig. 1).

Enumeration of *V. cholerae* attachment and invasion. 3×10^5 Caco-2 cells per well of 12-wells plate were cultured in two sets of triplicate for each experimental group. At the end of the secretome treatment, Caco-2 cells were infected with *Vibrio cholerae* in two doses of 5×10^6 CFU and 2×10^7 CFU. The bacterial attachment and invasion were evaluated according to our previous study (8).

Measurement of IL-6 and PGE2 cytokines and NO production. Caco-2 cells were treated with secretome of Mesenchymal Stem Cells for 48h and then exposed to 5×10^6 CFU *V. cholerae* for 2h. The IL-6 and PGE2 production was evaluated by ELISA assay according to our previous study with the application of mitomycin (10 µg/mL) for the prevention of bacterial growth (8). Nitric oxide production was also evaluated by Griess assay in collected supernatants after 12 h and 48 h of incubation (8).

Real time PCR analysis. The expression of the Ctx-B gene and the proinflammatory cytokines (IL-8, IL-1B, and TNF-α) were evaluated by Real time PCR in secretome treated Caco-2 cells in exposure to 5×10^6 CFU *V. cholerae* for 4h. Data was analyzed by the $2^{-\Delta\Delta Ct}$ algorithm and reported in Fold Regulation (FR). In data analysis, to investigate the effects of secretome and *V. cholerae* treatment separately, two types of control groups were used: 1. Caco-2 cells and 2. *V. cholerae* treated Caco-2 cells. The oligonucleotide sequences of primers used for real time PCR (11, 12) are reported in Table 1.

Apoptosis and necrosis assay. To investigate the effect of AD-MSCs secretome on the apoptosis rate of Caco-2 cells treated with *V. cholerae*, an AnnexinV-PI staining kit (BioLegend) was used. Caco-2 cells were treated with secretome for 48h and then exposed to two concentrations of 5×10^6 CFU and 2×10^7 CFU *Vibrio cholerae* for 2h. The apoptosis and necrosis rate were evaluated by AnnexinV-PI staining flowcytometry.

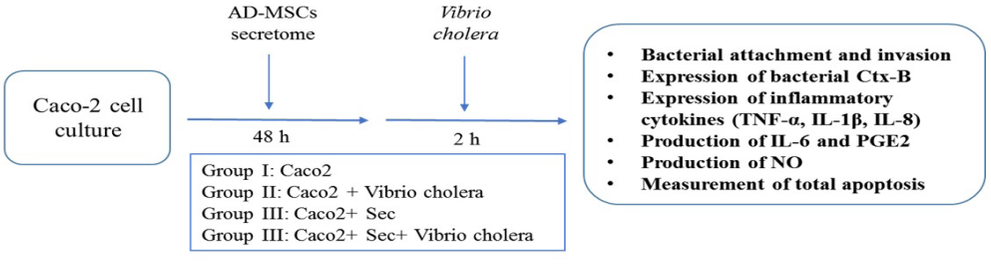

Fig. 1. Schematic representation of experimental groups and their treatment schedule.

Table 1. The oligonucleotide sequences of IL-1β, IL-8, TNF-α, and Ctx-β primers used for real time PCR.

	The primer oligonucleotide sequences (5'-3')	Gene
1	Forward primer: GCACGAT GCACCTGTACGAT Reverse primer: AGACATCACCAAGCTTTTTTGCT	IL-1β
2	Forward primer: AAACCACCGGAAGGAACCAT Reverse primer: GCCAGCTTGGAAGTCATGT	IL-8
3	Forward primer: CAGAGGGAAGAGTTCCCCAG Reverse primer: CCTTGGTCTGGTAGGAGACG	TNF-α
4	Forward primer: GGTTGCTTCTCATCATCGAACCAC Reverse primer: GATACACATAATAGAATTAAGGAT	Ctx-B
5	Forward primer: GATCATCAGCAATGCCTCC Reverse primer: TCCACGATACCAAAGTTGTC	GAPDH

RESULTS

AD-MSCs characterization. A homogeneous population of AD-MSCs with fibroblast like morphology is shown in Fig. 2A. Lipid droplets of adipogenic differentiation and calcium deposits of osteogenic differentiation of AD-MSCs at passage three were confirmed using ORO and AR staining, respectively (Figs. 2B and C). The immunophenotyping assay showed that AD-MSCs were negative for CD45 cell surface marker (0.05 %), but CD105, CD90, CD73, and Sca-1 surface markers were expressed at the mean percent of 76, 52, 61, and 74%, respectively (Fig. 2D).

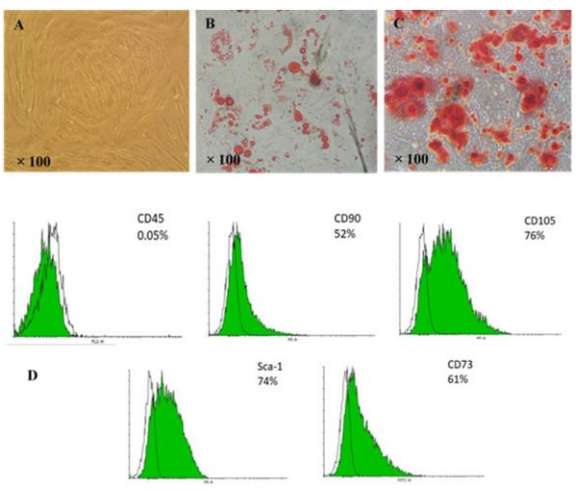

Fig. 2. AD-MSCs characterization. AD-MSCs with Fibroblast like morphology (A). Differentiation of AD-MSCs to adipocyte (B) and osteocytes (C). The immunophenotyping analysis of AD-MSCs (D).

Morphological evaluation of secretome treated Caco-2 cells. Caco-2 cells in exposure to AD-MSCs secretome (Fig. 3D) and without any treatment (Fig. 3A) were interconnected and polyhedral. Whereas, in the treatment with bacteria, the attachment of the cells to each other and the floor is loosened and weakened their close association with each other (Figs. 3B, C, E, and F). Exposure to Caco-2 cells with a higher concentration of *V. cholerae* (2×10^7) causes higher morphological change and cell dissociation.

The extent of invasion and attachment of *V. cholerae*. During the treatment of Caco-2 cells with 2×10^7 CFU *V. cholerae* in the well, the invasion of bacteria to Caco-2 cells was 13.14 ± 5.17, which decreased to zero in secretome treated cells ($P < 0.0001$). In the group of 5×10^6 CFU, *V. cholerae* had no invasion to the test and control wells. Analysis of *V. cholerae* attachment to cells also showed that in the group of 2×10^7 CFU, the rate of bacterial attachment was 4,193,529 ± 241,886, which in secretome treatment decreased by 61.23% to 1,625,417 ± 219,849 ($P < 0.0001$). Bac-

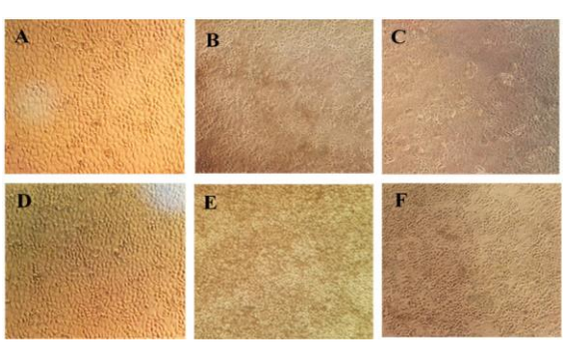

Fig. 3. Treatment of Caco2 cell line with AD-MSCs secretome and experimental groups after 48 h (×100). (A) Caco2 cells, (B) Caco2 cells + *V. cholerae* (5×10^6 CFU), (C) Caco2 cells + *V. cholerae* (2×10^7 CFU), (D) secretome treated Caco2 cells, (E) secretome treated Caco2 cells + *V. cholerae*, (5×10^6 CFU), (F) secretome treated Caco2 cells + *V. cholerae* (2×10^7 CFU).

terial attachment to Caco-2 cells in the group of 2 × 10^7 CFU *V. cholerae* was 3,470,000 ± 145,559 which in treatment with secretome decreased by 64.73% to 1,223,787 ± 145,804 (P <0.0001) (Fig. 4 and Table 2).

Evaluation of IL-6 and PGE2 production. The IL-6 production in Caco-2 cells after 48 hours of incubation was 568.51 ± 63.26 pg/mL which was decreased to 23.92 ± 6.68 with *V. cholerae* exposure (P <0.0001). The IL-6 production of secretome treated Caco-2 cells (814.33 ± 14.99 pg/mL) was significantly higher than Caco-2 cells (P <0.0001) and significantly (P= 0.01) reduced (81.33%) to 128.1 ± 37.6 pg/mL by *V. cholerae* exposure (Fig. 5A).

The PGE2 production from Caco-2 cells was 101.4 ± 18.97 pg/mL and reached to 121.1 ± 19.95 pg/mL in treatment with *V. cholerae* (P = 0.07). The PGE2 production in secretome treated Caco-2 cells was also 71.05 ± 5.56 pg/mL, which was lower (P = 0.27) than in Caco-2 cells. The PGE2 production in secretome treated Caco-2 in exposure to *V. cholerae* (58.01 ± 4.58 pg/mL) showed a significant 51.97 % decrease compared to Caco-2 cells treated with *V. cholerae* (P = 0.0007) (Fig. 5B).

No production. The NO production from Caco-2 cells was 5.42 ± 1.2 and 9.06 ± 2.73 µM respectively in 12h and 48h supernatants. The *V. cholerae* exposure increased the mean NO production respectively to 8.28 ± 1.12 µM (P=0.01) and 16.63 ± 2.32 µM (P=0.0002). The mean NO production in 12h and 48h supernatants of secretome treated Caco-2 cells was 4.69 ± 0.73 (P=0.75) and 9.5 ± 1.77 (P=0.98) µM respectively, which was not significantly different from Caco-2 cells. The mean NO production in Caco-2 cells treated with secretome and *V. cholerae* in 12h and 48h supernatants was 5.42 ± 2.18 and 9.51 ± 1.22 µM, which decreased by 34.3% (P= 0.02) and 42.81% (P= 0.0007), respectively compared to Caco-2 cells treated with *V. cholerae* (Fig. 6).

Evaluation of Ctx-β, IL-8, IL-1β, and TNF-α gene expression. As shown in Fig. 7, the relative gene expression of the Ctx-B in Caco-2 cells increased by 3.07 ± 0.56 fold during *V. cholerae* treatment. The expression of Ctx-B in Caco-2 cells treated with secretome and *V. cholerae* also decreased by 1.59 ± 0.78 fold compared to Caco-2 cells (P= 0.02). The relative gene expression of IL-8, IL-1β, and TNF-α inflammatory cytokines was increased by 2.27 ± 0.31, 4.76

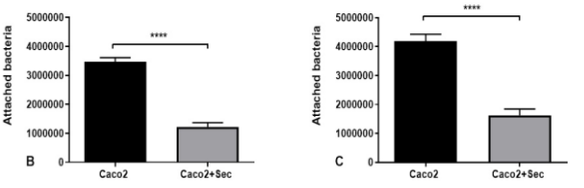

Fig. 4. *Vibrio cholerae* attachment to secretome (Sec) treated Caco-2 cells in exposure to 5×10^6 (A) and 2×10^7 (B) CFU

Table 2. Calculation of *Vibrio cholerae* (5×10^6 CFU and 2×10^7 CFU) attachment and invasion to Caco-2 epithelial cells

Bacteria CFU	Groups	Attachment + Invasion (Mean ± SD)				Invasion (Mean±SD)	Attachment (Mean ± SD)
		×10,000 dilution	×20,000 dilution	×30,000 dilution	Mean of dilutions		
5×10^6 CFU	Caco-2	3,756,000 ± 247,850	2,966,667 ± 244,404	-	3,470,000 ± 145,559	0	3,470,000 ± 145,559
	Caco-2 + secretome	1,045,455 ± 103,088	1,417,143 ± 254,146	1,357,500 ± 159,351	1,223,788 ± 145,804	0	1,223,788 ± 145,804
	P-value					-	<0.0001
2×10^7 CFU	Caco-2	4,191,875 ± 358,426	4,574,000 ± 151,261	3,930,000 ± 427,734	4,193,542 ± 241,886	13.14 ± 5.17	4,193,529 ± 241,886
	Caco-2 + secretome	1,575,000 ± 188,072	1,835,000 ± 361,702	1,260,000 ± 125,499	1,625,417 ± 219,849	0	1,625,417 ± 219,849
	P-value					<0.0001	<0.0001

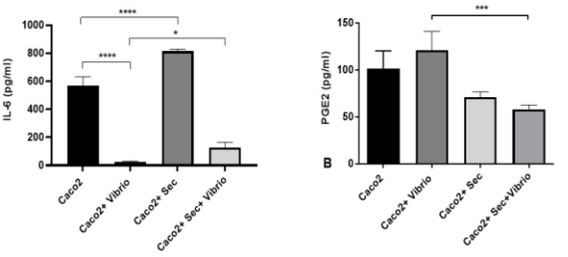

Fig. 5. IL-6 (A) and PGE_2 (B) production in 48h supernatants of secretome (Sec) treated Caco-2 in response to *Vibrio cholerae*

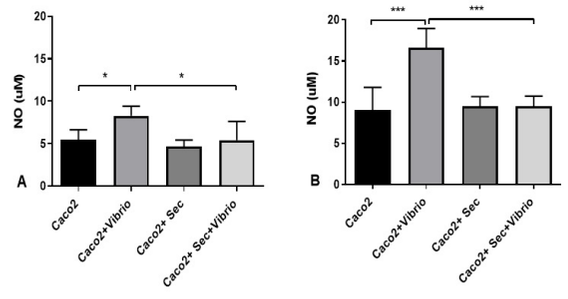

Fig. 6. NO production in 12h (A) and 48h (B) supernatants of secretome (Sec) treated Caco-2 cells in response to *Vibrio cholerae.*

± 1.52, and 139.6 ± 5.47 fold respectively in Caco-2 cells treated with *V. cholerae*. The expression of these cytokines in cells treated with secretome respectively showed a 1.12 ± 0.17 fold decrease, 2.22 ± 0.05 fold increase, and 14.65 ± 2.15 fold increase.

The expression of IL-8, and IL-1β cytokines in *V. cholerae* and secretome treated Caco-2 cells decreased respectively by 1.08 ± 0.08 (P= 0.001), and 4.79 ± 0.09 fold (P=0.003) compared to Caco-2 cells, but the TNF-α expression increased by 8.96 ± 0.88 fold. Secretome cause the significant reduction of TNF-α after *V. cholerae* exposure (P< 0.0001).

Apoptosis and necrosis. The apoptosis rate of Caco-2 cells was % 3.1 ± 1.63, which in the treatment with 5×10^6 CFU *V. cholerae* reached to %45.58 ± 1.16 (P <0.0001). The apoptosis rate of Caco-2 cells treated with secretome and *V. cholerae* was also % 33.22 ± 2.86 which showed a significant decrease of 12.36% in comparison with Caco-2 cells treated with *V. cholerae* alone (P <0.0001). According to Fig. 8B, the apoptosis rate of Caco-2 increased from % 3.71 ± 1.33 to % 46.03 ± 1.17 (P <0.0001) in exposure to 2×10^7 CFU *V. cholerae*. The mean apoptosis of Caco-2 cells treated with secretome and *V. cholerae* was also %

A

	Caco-2 + Vibrio	Caco-2 + Sec	Caco-2 + Sec + Vibrio
Ctx-B	3.07 ± 0.56	-	-1.59 ± 0.78
TNF-α	139.6 ± 5.47	14.65 ± 2.15	8.96 ± 0.88
IL-1β	4.76 ± 1.52	2.22 ± 0.05	-4.79 ± 0.09
IL-8	2.27 ± 0.31	-1.12 ± 0.17	-1.08 ± 0.08

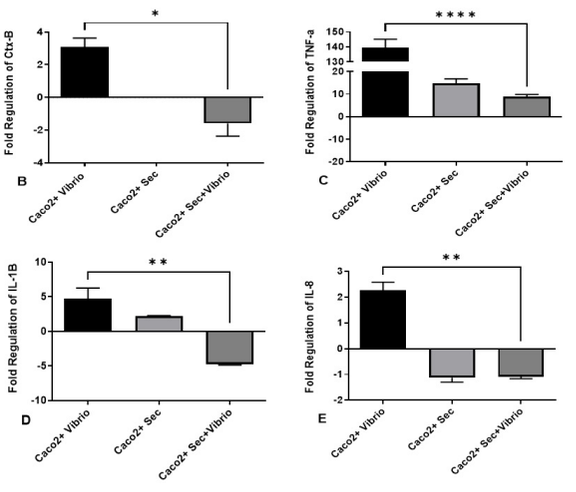

Fig. 7. The Fold Regulation in expressions (A) of Ctx-B (B), TNF-a (C), IL-1B (D) and IL-8 (E) gene in Caco-2 cells treated with secretome (Sec) and *Vibrio cholerae* compared to control groups.

21.76 ± 2.95 which showed a significant decrease of 24.27% in comparison with Caco-2 cells treated with *V. cholerae* alone (P <0.0001) (Fig. 8A).

The necrosis rate of Caco-2 cells was % 1.35 ± 1.03, which in exposure to 5×10^6 CFU *V. cholerae* reached 6.42 ± 3.17 (P= 0.005). The necrosis rate of Caco-2 cells treated with secretome and *V. cholerae* was also %7.58 ± 2.09 which does not have significant differences with *V. cholerae* treated Caco-2 cells (P= 0.70) (Fig. 8B). In exposure to the concentration of 2×10^7 CFU *V. cholerae*, the necrosis rate of Caco-2 increased from % 1.57 ± 1.07 to % 43.13 ± 2.76 (P= 0.03). The mean necrosis of Caco-2 cells treated with secretome and *V. cholerae* was also % 44.68 ± 27.57 which does not have significant differences with *V. cholerae* treated Caco-2 cells (P= 0.99).

DISCUSSION

The discovery of mesenchymal stem cells in the 1960s and their self-renewal and multipotent differ-

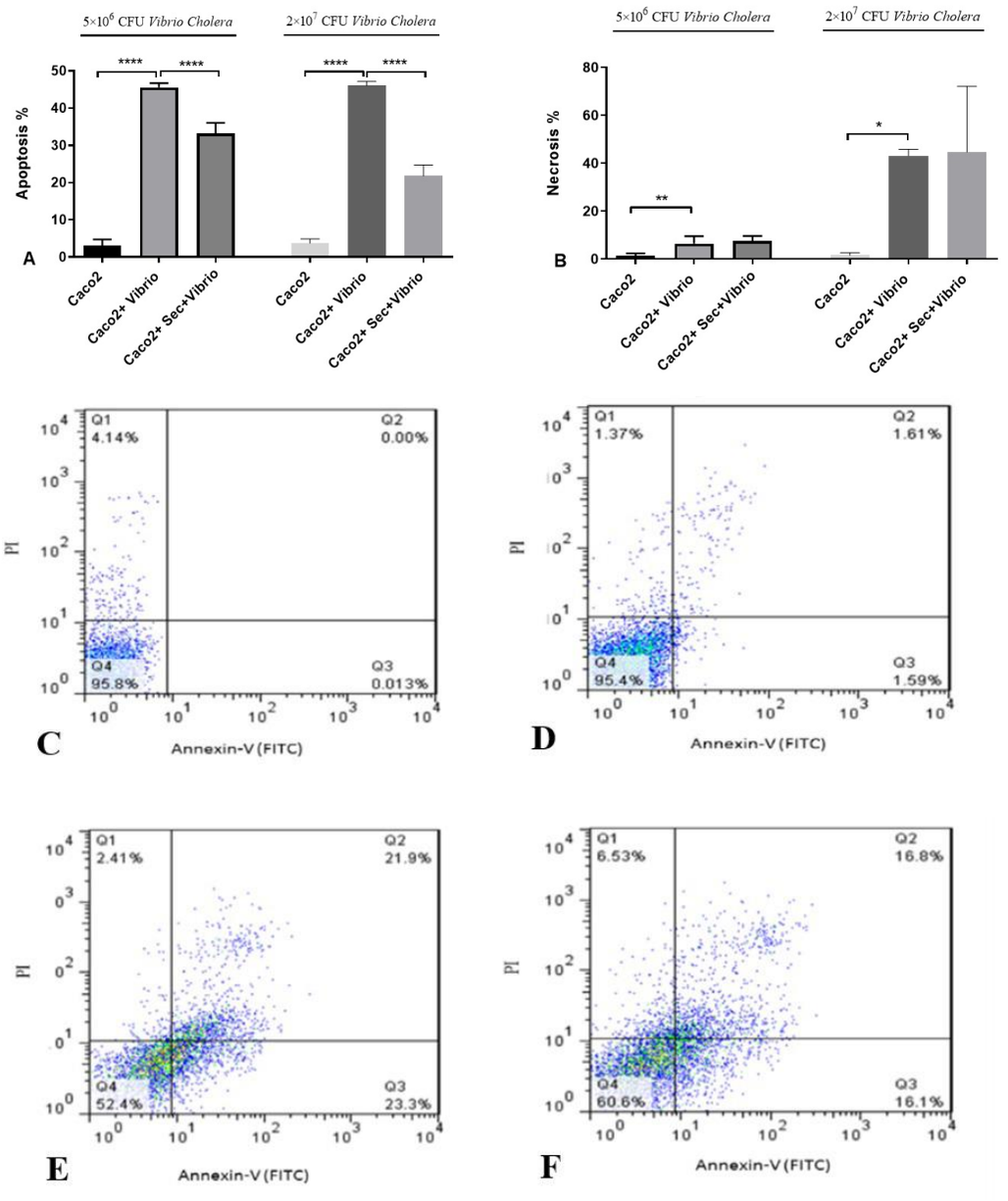

Fig. 8. The evaluation of the effect of AD-MSCs secretome on the apoptosis and necrosis rate in response to *V. cholerae* infection. (A) The Mean percentage of apoptosis in exposure to *V. cholerae*. (B) The Mean percentage of necrosis in treatment with *V. cholerae*. (C) The flow cytometry diagrams of apoptosis and necrosis in unstained Caco-2 cells. (D) The flow cytometry diagrams of apoptosis and necrosis in Caco-2 cells stained with Annexin (FITC) and PI. (E): The flow cytometry diagrams of apoptosis and necrosis in Caco-2 cells treated with *V. cholerae*. (F): The flow cytometry diagrams of apoptosis and necrosis in Caco-2 cells treated with *V. cholerae* and secretome (Sec).

entiation potential opened a new window in the treatment of disease based on tissue repair and regeneration (13). In the following years, with the determination of the immunomodulatory role of MSCs through secretion of soluble factors including TGF-β, IL-10, HGF, sHLA-G5, TSG-6, and so on they were considered in the treatment of autoimmune and inflammatory disease (14, 15). Finally, the identification of microbial ligands by pathogen recognition receptors expressed on MSCs, secretion of antimicrobial peptides, and sensing of inflammatory conditions of microenvironment, promoted their application in infectious diseases (16, 17).

Positive effects of MSC therapy in the treatment

of some infectious diseases including viral, parasitic, fungal, and bacterial origin have already been shown in preclinical studies (18, 19). In addition, MSC therapy has also achieved significant success in clinical trials of infectious disease such as sepsis (20). MSCs help to improve infection and its complications mainly by reducing microbial load, repairing damaged tissue, and enhancing antimicrobial immune responses (21). While the role of MSC therapy in bacterial infections such as sepsis, pneumonia, and tuberclosis has been well studied, gastrointestinal bacterial infections such as cholera and shigellosis have received less attention. Treatment of intestinal infections is important for several aspects: 1) Most intestinal infections do not have vaccines and every year 3 to 6 million children die from gastroenteritis, 2) antibiotics are the main therapeutic strategy for gastrointestinal infections, which lead to the formation of antibiotic resistance in the population, 3) Inflammation caused by gastrointestinal infections changes microbial flora and increases the susceptibility to inflammatory diseases like inflammatory bowel disease (IBD) (22).

For this purpose, the current study was designed to assess *in vitro* effect of MSCs derived supernatant on *V. cholerae* interaction with Caco-2 cells as representative of human intestinal epithelial cells (23). The first step in *V. cholerae* pathogenicity is the bacterial attachment to the epithelial cells. Therefore reducing bacterial adherence and invasion of the host cell can be an effective therapeutic approach (24). Different vaccines elicited antibody formation against *V. cholerae* toxin coregulated pilus (TCP), antimicrobial nanocomposites and natural drugs like thymoquinone are among treatments based on inhibition of cell attachment (25). A recent study by the present team demonstrated the anti-biofilm activity of MSCs secretome against *V. cholerae* (21) but the effects of MSCs secretions on bacterial attachment and invasion to the host cell were not investigated. The findings of the present study showed that the supernatant of MSCs can reduce *V. cholerae* attachment to the Caco-2 epithelial cell surface by up to 60%. Two possible mechanisms for inhibiting *V. cholerae* attachment by MSC secretion are suggested. The first mechanism is the expression of high levels of the indoleamine 2,3-dioxygenase (IDO) by human MSCs, which leads to the consumption of tryptophan in the environment. Indole is one of the important mediators in regulating the pathogenesis of *V. cholerae*, which results from the catabolism of tryptophan by the enzyme tryptophanase encoded in *V. cholerae* (26). The presence of indole in the early phase of infection encourages biofilm formation and bacterial colonization on the epithelial cells' surfaces (27). Therefore, limiting the source of indole production will prevent bacteria from accumulating together and attaching to the surface. The high secretion of tissue inhibitor matrix metalloproteases (TIMPs) by human MSCs can be the second reason for reduced *V. cholerae* attachment and invasion of epithelial cells (9, 28). Production of extracellular proteolytic enzymes by *V. cholerae*, including metalloproteinase are among the pathogenic activities of bacteria. These enzymes by breakdown of tight junction proteins allow bacteria to attach to the epithelial surface (29, 30). Thus, inhibition of metalloproteases by TIMPs inhibits the access and attachment of bacteria to the Caco-2 cells.

By reducing the *V. cholerae* colonization on the Caco-2 epithelial cells surface, the expression of chlorotoxin and the PGE2 production decreased. In our previous study, direct co-culture of MSCs decreased the expression of chlorotoxin in epithelial cells. But it was not effective on PGE2 production. Therefore, it appears that AD-MSCs soluble factors have a stronger effect on reducing bacterial colonization, chlorotoxin expression, and PGE2 production (24). PGE_2 plays a key role in the pathogenesis of *V. cholerae* in the recruitment of inflammatory cells, intensifying destructive inflammatory responses. Probably, the PGE2 reduction will be effective in inhibiting inflammatory responses and epithelial barrier destruction. Lee et al. reported that AD-MSCs can inhibit the excessive production of PGE_2 (by decreasing COX-2 enzyme activity) in a mouse model of pneumonia, and led to the improvement of the phagocytic function of host macrophages and bacterial elimination (31).

In the present study, the MSCs secretome reduced the expression of IL-8, IL-1β, and TNF-α inflammatory cytokines as well as NO production. Several previous studies have also reported the potent immunomodulatory effects of MSCs in reducing the level of pro-inflammatory cytokines caused by bacterial infections. Yuan et al. showed the anti-inflammatory effect of these cells on bacterial infection caused by methicillin-resistant *Staphylococcus aureus* (32). Chenari et al. also indicated that MSCs modulate the inflammatory responses to BCG in-

fection and decrease the secretion of TNF-α and IL-1β in the alveolar fluid. They also reported that the TNF-α/IL-10 ratio was decreased after successive secretome treatment (17). Hazrati et al. reported that the combination of MSC-derived exosomes and imipenem can effectively reduce the secretion of NO, TNF-α, and IL-1β inflammatory mediators in the *E. coli*-infected HepG2 cells (16). Eshghi et al. also reported that MSC-derived exosomes can decrease the levels of IL-1β and TNF-α inflammatory cytokines and ameliorate the subsequent destructive effects of inflammation caused by sepsis in a mouse model of LPS-induced systemic inflammation (33). Khosrojerdi et al. also reported that the mesenchymal stem cell-derived exosomes combined with imipenem can ameliorate inflammatory responses and liver damage in a sepsis mouse model (34). According to the decrease in the TNF-α, IL-1β, and IL-8 as well as NO production, it appears that the AD-MSCs secretome inhibits the NF-κB signaling pathway (35).

In the present study, the MSC secretome caused to increase in the expression of TNF-α and IL-1β inflammatory cytokines in uninfected Caco-2 cells. However, the expression of the cytokine transcripts decreased in secretome and *V. cholerae* treated Caco-2 cells. This is probably due to the dual function of mesenchymal stem cells in inflammatory and non-inflammatory conditions and also in different phases of infection (36). However, it is necessary to further investigate the activation or inhibition of the inflammatory cytokines production and also the signaling pathways involved in Caco-2 cells treated with secretome in future studies.

In the present study, the AD-MSCs secretome led to an increase in the production of IL-6 from epithelial cells and a decrease in their apoptosis. It appears that the soluble factors of AD-MSCs can reduce the apoptosis of epithelial cells by increasing the expression of IL-6 and anti-apoptotic genes (Bcl-xL, Mcl-1, cIAP-2, and Bcl-3). By increasing the proliferation and differentiation of epithelial cells, it helps to protect the integrity of the intestinal epithelial barrier (37).

IL-6 plays a key role in protecting the epithelial integrity. The inhibition of IL-6 production intensifies the infection-induced apoptosis in intestinal clonal epithelium and tissue injury occurs. In fact, IL-6 plays a critical role in protecting the integrity of the physical barrier of the intestine by preventing the formation of ulcers, which is a good breeding ground for bacteria (38). In addition to the anti-apoptotic effect of IL-6, previous studies have also shown that the MSCs soluble factors can lead to a decrease in apoptosis by reducing the activation of caspase 3 and 7 (39). The presence of IGF-1 in the secretions of MSCs can also help increase the viability of epithelial cells by activating the PI3K-Akt signaling pathway (40).

CONCLUSION

Soluble factors of AD-MSCs reduced *V. cholerae* colonization on the surface of Caco-2 epithelial cells. With the reduction of *V. cholerae* colonization, the penetration of chlorotoxin into the cytoplasm and the production of PGE2 also decreased, which caused a decrease in the production of IL-8, IL-1β, and TNF-α inflammatory cytokines and nitric oxide metabolites. Also, the production of soluble mesenchymal stem cell factors led to an increase in the production of IL-6 from Caco-2 epithelial cells and decreased their apoptosis rate. Therefore, it appears that the soluble factors of AD-MSCs can play a very effective role in preventing the destruction of the epithelial barrier and saving patients from hypovolemic shock.

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