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Immunomodulatory Effects of Adipose-derived Mesenchymal Stem Cells on Epithelial Cells Function in Response to *Vibrio cholera* in a Co-culture Model

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ABSTRACT

Inflammation-induced by the interaction of the *Vibrio cholerae* with the epithelial cells is considered as a main cause of bacteria spreading through the gastrointestinal tract and its consequences. Because of the immunomodulatory and antibacterial properties of adipose-derived mesenchymal stem cells (AD-MSCs), this study aimed to investigate the effect of AD-MSCs on the interaction of the bacterial-epithelial cell.

Caco-2 differentiated to intestinal epithelial cells co-cultured with AD-MSCs in a 1:1 ratio of the surface area of six-well plates, for 48 hours. After exposure to *Vibrio cholerae*, bacterial attachment and internalization were evaluated. Secretions of interleukin (IL) -6, prostaglandin E2 (PGE2), and nitric oxide (NO) were also measured using ELISA, and Griess assay, respectively. In addition, the expression of *chloratoxin (Ctx-β)* and inflammatory cytokines such as *TNF-a*, *IL-1β*, and *IL-8* were evaluated by real-time polymerase chain reaction (RT-PCR). The rate of apoptosis was also evaluated by Annexin V-PI flow cytometry.

Bacterial attachment and Ctx-β expression were significantly reduced in the co-culture group compared to the *Vibrio cholerae*-exposed Caco-2. IL-6 and PGE2 secretion increased in the co-culture group. NO, was also slightly reduced in exposure to *Vibrio cholerae*. An elevated level of bacterial internalization was observed in the co-culture group compared to the Caco-2 cells leading to an increase in the expression of pro-inflammatory cytokines. The rate of apoptosis was also increased significantly.

Cell-to-cell contact of AD-MSCs and Caco-2 promoted inflammatory responses and disruption of the epithelium barrier by enhancing bacterial invasion. This may be due to the high expression of surface matrix metalloproteinases on MSCs.

Keywords: Apoptosis; Caco-2 cells; Interleukin-6; Mesenchymal stem cells; Vibrio cholerae; Virus internalization

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INTRODUCTION

Cholera is an acute and dangerous intestinal diarrhea that causes death if hypovolemic shock occurred. According to World Health Organization

(WHO), 7 million cholera cases worldwide causing 100,000 to 130,000 deaths annually.[1] *Vibrio cholerae*, as a causative agent of cholera, is a non-invasive enteric gram-negative bacteria that exerts its pathological effects by Cholerae toxin (CT) production. The produced CT induces cAMP and PGE2 production, which causes to recruit of inflammatory cells such as neutrophils, mast cells, and macrophages. The recruited cells respond to infection by lactoferrin and myeloperoxidase secretion in the excretory liquid and production of inflammatory cytokines like TNF-α, interleukin (IL)-1, and IL-6.[2,3] These events lead to induce apoptosis, loss of defensive function of the epithelial barrier, and fluids excretion from the gastrointestinal tract. If epithelial barrier destruction and fluid loss are not suppressed, the disease will end in death.[3]

Today, Electrolytes and antibiotics are used in the treatment of patients with cholera. *Vibrio cholera* strains have become resistant to major antibiotic classes such as tetracyclines and macrolides. Cholera treatment is severely restricted especially in children and developing countries and causes many deaths.[1] Therefore, the use of alternative antibacterial compounds is critical. Efficient drugs with the combined effects of antibacterial, anti-inflammatory, and epithelial barrier repairing probably have an increasing effect on the prevention and treatment of cholera.

Mesenchymal Stem Cells (MSCs) present the combined antibacterial, anti-inflammatory, and epithelial barrier repairing effects. MSCs present their antibacterial effects by secreting antibacterial compounds of LL-37, hCAP-18, and lipocalin 2 and control bacterial growth and proliferation. Previous Vivo studies have also shown that MSCs are useful in the treatment of bacterial sepsis and increase bacterial clearance.[4] MSCs also play an important role in regulating immune responses depending on their microenvironment. They show strong anti-inflammatory effects in stimulation with inflammatory signals of microbial products.[5,6] MSCs have an effective role in epithelial barrier repair and ameliorate tissue damage by regulating immune responses.[7,8] Thus, MSCs appear to be able to reduce the inflammatory responses and pathological complications caused by *Vibrio cholerae* in the epithelial barrier. The present study was performed to investigate the immunomodulatory effects of MSCs on Caco-2 epithelial cell line in exposure to *Vibrio cholerae*.

MATERIALS AND METHODS

MSCs Culture and Characterization

MSCs isolated from human abdominal fat by enzymatic digestion method. Primary MSCs were cultured in a DMEM medium (Biosera, Germany) supplemented with 10% FBS (Gibco, USA). The cells were passaged after reaching 90% confluence and used in passage 3 in subsequent experiments. To confirm the purity of the isolated adipose-derived mesenchymal stem cells (AD-MSCs), CD45, CD34, CD29, CD90, CD73, and CD105 cell surface markers, were analyzed by flow cytometry method. AD-MSCs were cultured in adipogenic and osteogenic differentiation media for 21 days and stained by Alizarin Red or Oil Red O stains to determine the adipocyte and osteocyte differentiation potential, respectively.[9] This study was approved by the Medical Ethics Committee of Tarbiat Modares University (Code: IR.MODARES.REC.1397.009).

Co-culture of AD-MSCs with Caco2 Cells and Experimental Groups

Co-culture was established in the way that each cell covered 50% of the well surface area. In the co-culture wells, 4×10^4 AD-MSCs were seeded in 12 wells plates and incubated to adhere. After 12 hours, 1.5×10^5 Caco2 cells were added to the same wells and incubated for 48 hours to make a co-culture. In control wells, 8×10^4 AD-MSCs and 3×10^5 Caco2 cells were cultured. The experimental groups were then treated with *Vibrio cholerae* (ATCC14035). Experimental groups were defined as AD-MSCs, Caco2 cells, co-culture of AD-MSCs and Caco2 cells, AD-MSCs + *Vibrio cholerae,* Caco2 cells + *Vibrio cholerae,* and co-culture of AD-MSCs and Caco2 cells + *Vibrio cholerae.*

Vibrio cholerae Attachment and Internalization

After 48 hours of co-culture, experimental groups were infected with 5×10^6 and 2×10^7 CFU of *Vibrio cholerae* for 2 hours. All infected wells were washed three times with PBS (Merck, Germany) to remove unattached bacteria. A triplicate set of wells for each experimental group were lysed with 0.01% Triton-X 100 (Sigma, USA) and cultured onto BHI agar (Sigma, USA) at different serial dilutions and indicating the total number of attached and invaded bacteria. Another triplicate set of infected cells were treated with 100 µg/mL of gentamicin (Sigma, USA) for 1 hour to kill attached bacteria. Then the cells were lysed with 0.01%

Triton-X 100 in PBS and cultured onto BHI agar at different serial dilutions. The mean number of colonies indicates the number of bacteria invading the cells. The number of attached bacteria was calculated by subtracting the number of internalized bacteria from the total number of bacteria.[10,11]

Measurement of IL-6, PGE2, and NO Production

Caco-2 cells were co-cultured directly with AD-MSCs for 48 hours and then exposed to 2×10^7 CFU *Vibrio cholerae* for 2 hours. After washing and removing the bacteria, cells were treated with mitomycin 10 µg/mL (Sigma, USA) for another 2 hours to prevent the growth of the attached bacteria. Subsequently, the new DMEM medium was replaced with mitomycin-containing media. The supernatants were collected after 48 hours of incubation and stored at -70°C until measurement. Concentrations of IL-6 and PGE2 were measured using R&D (USA) and Invitrogen (USA) commercial kits, respectively.[12] Griess test was also used to assay the NO production. According to this method, the collected supernatant was mixed 1:1 to Griess reagent (Cibbiotech, Iran) and their optical absorption was calculated in 540 nm. The NO production was reported based on the standard curve of sodium nitrite.[13]

Real-time Polymerase Chain Reaction (RT-PCR) Analysis

Caco-2 cells were co-cultured directly with AD-MSCs for 48 hours and then exposed to 5×10^6 CFU *Vibrio cholerae* for 4 hours. After washing and removing the bacteria, another 2 hours' incubation was done in culture media at 37°C. The gene expression of the *Ctx-B* subunit of chloratoxin and the pro-inflammatory cytokine (*TNF-α*, *IL-1B*, and *IL*-8) gene expressions were evaluated by real-time PCR. *GAPDH* gene was used as the internal control. The nucleotide sequence of the related primers is reported in Table 1. The fold regulation (FR) and the $2^{-\Delta\Delta Ct}$ algorithm were considered to report the relative expression level of each gene.[14] To investigate the effect of bacterial exposure on the gene expression, the ratio of gene expression of MSC+V, Caco-2+V, and MSC*Caco-2+V was calculated to MSC, Caco-2, and MSC*Caco-2, respectively. In addition, the ratio of the gene expression of MSC*Caco-2+V to MSC+V and Caco-2+V was determined to show the co-culture effects.

Apoptosis

AnnexinV-PI staining kit (BioLegend, USA) was used to investigate the effect of direct contact of AD-MSCs on the apoptosis of Caco-2 cells in exposure to *Vibrio cholerae*. AD-MSCs were co-cultured with a Caco-2 cell line for 48hours and then exposed to 5×10^6 CFU removing the bacteria, another 1-hour incubation was done in culture media at 37°C. Cells were isolated using 0.25% trypsin-EDTA (Sigma, USA) and fluorescent intensity of AnnexinV and PI was determined; using the FACS CanII instrument according to the manufacturer's instructions.[16]

Table 1. The sequences of primers used in real-time polymerase chain reaction (RT-PCR) assay.

Primer	Primer Sequence (5'-3')	Ref
Ctx-β	F: 5′-GGTTGCTTCTCATCATCGAACCAC-3′ R: 5′-GATACACATAATAGAATTAAGGAT-3′	15
TNF-α	F: 5′-CAGAGGGAAGAGTTCCCCAG -3′ R:5′-CCTTGGTCTGGTAGGAGACG-3′	10
IL-8	F: 5′-AAACCACCGGAAGGAACCAT-3′ R:5′-GCCAGCTTGGAAGTCATGT-3′	
IL-1β	F: 5'- GCACGATGCACCTGTACGAT-3' R: 5'-AGACATCACCAAGCTTTTTTGCT-3'	
GAPDH	F: 5'-GATCATCAGCAATGCCTCC- 3' R: 5'-TCCACGATACCAAAGTTGTC- 3'	

Statistical Analysis

Statistical analysis of data was performed using t-test and ANOVA in GraphPad Prism 8.0.2 software. The significance level was considered less than 0.05 (p<0.05) and the data were expressed as Mean±SD.

RESULTS

Characterization of AD-MSCs

A homogeneous population of AD-MSCs with fibroblast-like morphology (Figure 1A) was isolated from the human abdominal fat. AD-MSCs differentiation into adipocytes and osteocytes was confirmed by oil red oil and alizarin red staining, which shows fat droplets and calcium deposits, respectively. (Figure 1B and 1C). The flow cytometry analysis indicated that isolated and expanded AD-MSCs were negative for CD34 and CD45 cell surface marker, but CD29, CD90, CD73, and CD105 surface markers were expressed at mean percent of 99.96%, 62.5%, 71.2%, and 85.36% respectively (Figure 1D).

Morphological Study of Caco-2 Cells and AD-MSCs Co-culture

To evaluate the immunomodulatory effect of AD-MSCs, Caco2 cells were co-cultured directly with AD-MSCs for 48 hours. AD-MSCs and Caco2 cells were co-cultured in a manner that each of them covered 50% of the well plate area. As shown in Figure 2, in the co-culture group (Figure 2A), the Caco-2 cells are well attached to the AD-MSCs by the network formation, indicating close interaction of these cells with each other. In separate groups of Caco-2 cells (Figure 2B) and AD-MSCs (Figure 2C), the cells cover the entire surface of the wells.

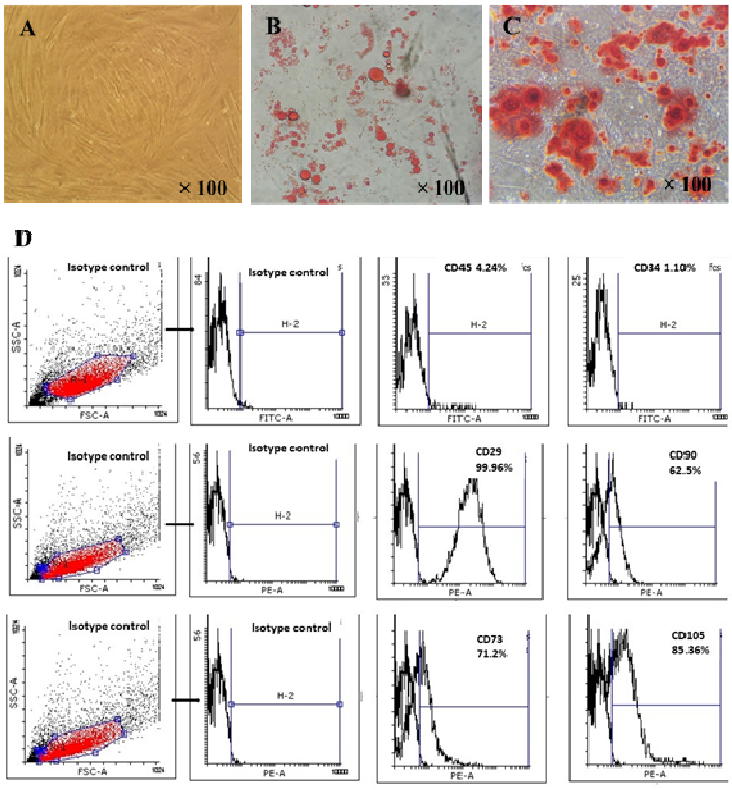

Figure 1. Characterization of human adipose-derived mesenchymal stem cells (AD-MSCs) by their morphology, differentiation, and phenotypic markers. AD-MSCs have a fibroblastic morphology (A). Oil Red O staining indicates lipid vacuoles and differentiation to adipocytes (B). Alizarin Red S staining indicates calcium mineralization and differentiation to osteocytes (C). The flow cytometry histograms indicate cell surface markers expression in human AD-MSCs (D).

The Attachment and Internalization of *Vibrio cholerae*

The bacterial attachment to the co-cultured group in exposure to 5×10^6 CFU *Vibrio cholerae* was 2'093'319±117'851 which increased by 48.72% (p=0.0005) compared to AD-MSCs (1'073'298±37'712) and decreased by 39.67% (p<0.0001) compared to Caco-2 cells (3'470'000±145'559) (Table 2 and Figure 3A). In exposure to 2×10^7 CFU *Vibrio cholerae*, the mean attachment of *Vibrio cholerae* to the co-culture group was 2'489'948±560580, which was significantly reduced by 40.62% (p<0.0001) compared to Caco-2 cells (4'193'529±241'886) and increased by 14% (p=0.1686) compared to AD-MSCs (2'141'187 ±136'955) (Figure 3B).

The bacterial internalization to AD-MSCs and Caco-2 cells in exposure to 5×10^6 CFU *Vibrio cholerae* respectively were 35.33±6.5 (p<0.0001) and zero (p=0.0002); which were significantly different from the co-cultured group (14±3.26) (Table 2, Figure 3C). In exposure to 2×10^7 CFU *Vibrio cholerae*, the internalization rate of *Vibrio cholerae* to the co-culture group (52.5±3.53) decreased by 17% (p=0.0824) compared to AD-MSCs (63.25±5.37) and increased by 74.97% (p<0.0001) compared to Caco-2 cells (13.14±5.17) (Figure 3D).

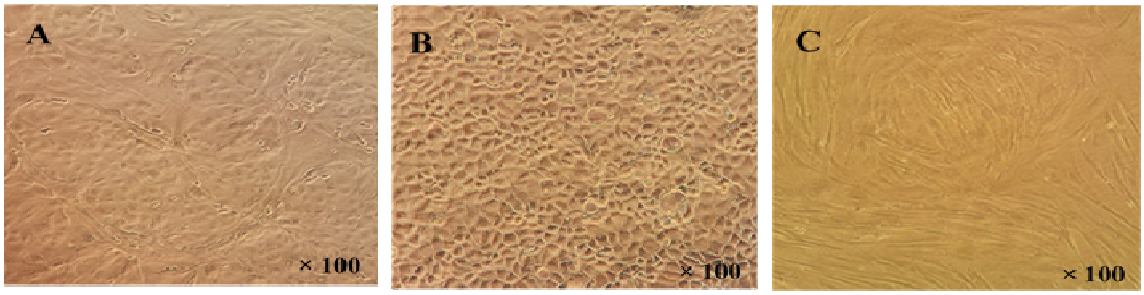

Figure 2. Co-culture of adipose-derived mesenchymal stem cells (AD-MSCs) and Caco-2 cell line after 48 hours of incubation (×100). (A) Co-culture of AD-MSCs and Caco-2 cell line; (B) Caco2 cells; (C) AD-MSCs.

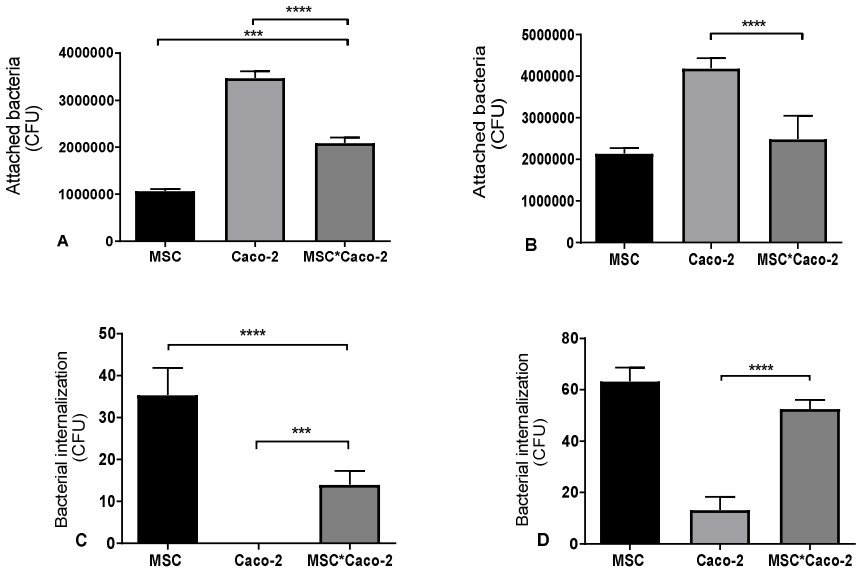

Figure 3. *Vibrio cholerae* attachment (A, B) and internalization (C, D) in exposure to 5×10^6 (A, C) and 2×10^7 (B, D) CFU *Vibrio cholerae*.

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Table 2. Bacterial attachment and internalization to experimental groups of adipose-derived mesenchymal stem cells (AD-MSCs), Caco-2, and Co-culture.

Bacteria CFU	Groups	Attachment + Internalization (Mean±SD)	Internalization		Attachment	
			Mean±SD	*p(compared to co-culture)*	Mean±SD	*p(compared to co-culture)*
5×10^6 CFU	AD-MSCs	1,073,333 ±37,712	35.33±6.5	**<0.0001**	1,073,298±37,712	**0.0005**
	Caco-2	3,470,000±145,559	0	**0.0002**	3,470,000±145,559	**<0.0001**
	Co-culture	2,093,333±117,851	14±3.26	-	2,093,319±117,851	-
2×10^7 CFU	AD-MSCs	2,141,250±136,955	63.25± 5.37	0.0824	2,141,187±136,955	0.1686
	Caco-2	4,193,542±241,886	13.14±5.17	**<0.0001**	4,193,529±241,886	**<0.0001**
	Co-culture	2,490,000± 560,580	52.5± 3.53	-	2,489,948±560,580	-

Evaluation of IL-6 Production

As shown in Figure 4A, the mean IL-6 production of AD-MSCs, Caco-2 cells, and co-culture group respectively were 820.35±68.06, 568.51±63.26, and 829.71±5.01 pg/mL after 48 hours; which was decreased in exposure to *Vibrio cholerae* and reached to 698.11±114.4 (p=0.1021), 23.92±6.68 (p<0.0001) and 294.53±40.91 (p<0.0001) pg/ml, respectively. IL-6 production in the co-culture group showed a 31.48 % significant increase compared to Caco-2 cells (p<0.0001), but there was no significant difference compared to AD-MSCs (p>0.99). The level of IL-6 in the *Vibrio cholerae* treated co-culture group also decreased by 57.8% compared to the *Vibrio cholerae* treated AD-MSCs (p<0.0001), but increased by 91.87 % compared to the *Vibrio cholerae* treated Caco-2 cells (p<0.0001).

Measurement of PGE2 Production

As shown in Figure 4B, the mean production of PGE2 in Caco-2 cells was 101.4±18.97 after 48 hours, which was increased to 120.8±20.18 in exposure to *Vibrio cholerae* (p=0.2787). The mean production of PGE2 in AD-MSCs cell and co-culture group respectively were 825±61.57 and 397.4±54.53 pg/mL; which was significantly decreased in exposure to *Vibrio cholerae* and reached 440.5±71.17 (p<0.0001) and 157.2±43.56 (p<0.0001) pg/mL. PGE2 production in the co-culture group showed a 74.48 % increase compared to Caco-2 cells (p<0.0001), but it was significantly reduced by 51.83 % compared to AD-MSCs (p<0.0001). The level of PGE2 in the *Vibrio cholerae* treated co-cultured group was significantly decreased by 64.31 % (p<0.0001) compared to the *Vibrio cholerae* treated AD-MSCs but increased slightly compared to the *Vibrio cholerae* treated Caco-2 cells (p=0.8657).

Measurement of NO Production

According to Figure 4C, The mean production of NO in AD-MSCs, Caco-2, and co-culture group respectively were 6.83±0.96, 8.26±1.91, and 4.55±0.72 µM after 48 hours; which was increased in exposure to *Vibrio cholerae* and reached to 8.71±0.95 (p=0.39), 17.73±0.85 (p<0.0001) and 15.74±0.27 (p<0.0001) µM, respectively. The mean NO production in the co-culture group was significantly reduced by 44.91% compared to Caco-2 cells (p=0.0027). The mean production of NO in the *Vibrio cholerae* treated co-cultured group was also decreased by 12.64% compared to *Vibrio cholerae* treated Caco-2 cells (p=0.39) and increased by 44.66% compared to *Vibrio cholerae* treated AD-MSCs (p<0.0001).

Evaluation of *Ctx-B* Gene Expression

As shown in Figure 5A and Table 3, the *Ctx-B* expression in the Caco-2 cell line in exposure to *Vibrio cholerae* increased by 3.06±0.57 fold (p=0.02) and slightly increased by 1.15±0.59 fold in AD-MSCs (p=0.99) and 1.4±0.12 fold increased in the co-culture group (p=0.85). The *Ctx-B* expression in the *Vibrio cholerae* treated co-cultured group was decreased by 2.15±0.27 fold (p=0.003) compared to *Vibrio cholerae* treated AD-MSCs and 3.57±0.94 fold (p=0.0005) decreased compared to *Vibrio cholerae* treated Caco-2 cells.

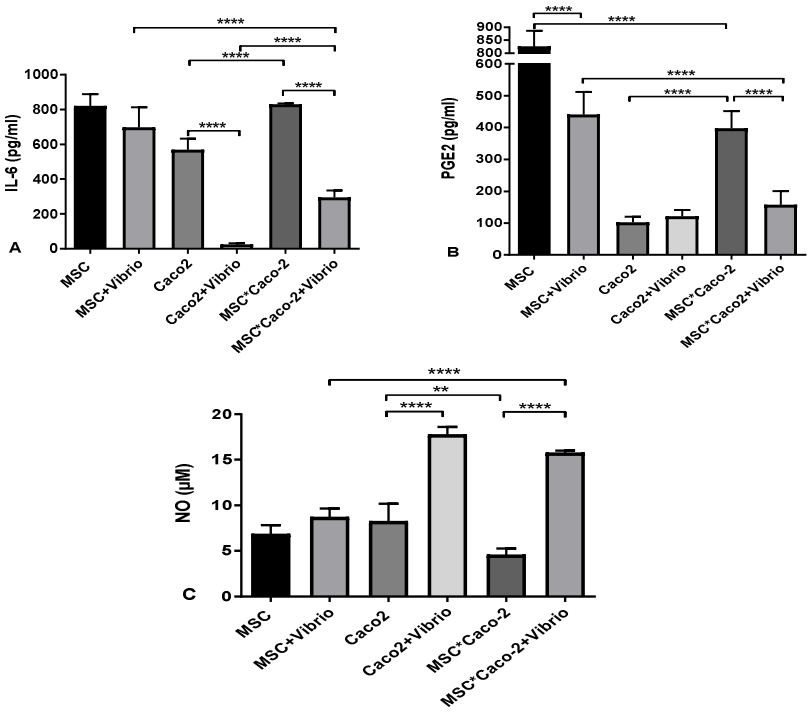

Figure 4. The production of interleukin (IL)-6 (A), prostaglandin E2 (PGE2) (B), and nitric oxide (NO) (C) after 48 hours of incubation in exposure to *Vibrio cholerae*.

Evaluation of the Gene Expression of Inflammatory Cytokines *TNF-α*, *IL-1β*, and *IL-8*

As shown in the Figures of 5B, 5C, 5D and Table 3, the gene expression of *TNF-α* in exposure to *Vibrio cholerae* increased by 33.94±0.16 fold in AD-MSCs ($p<0.0001$), 139.6±5.47 fold in Caco-2 cells ($p<0.0001$), and 31.89±0.77 in the co-cultured group ($p<0.0001$). The *TNF-α* expression in the *Vibrio cholerae* treated co-cultured group was increased by 1.8±0.04 fold (p=0.99) compared to *Vibrio cholerae* treated AD-MSCs and 6.39±0.9 fold (p=0.17) was decreased compared to *Vibrio cholerae* treated Caco-2 cells.

The gene expression of *IL-1β* in exposure to *Vibrio cholerae* increased by 13.02±1.14 fold in AD-MSCs (p=0.02), 4.76±1.51 fold in Caco-2 cells (p=0.60), and 19.1±2.43 fold in the co-cultured group (p=0.002). The *IL-1β* expression in the *Vibrio cholerae* treated co-cultured group was decreased by 17.76±3.13 fold (p=0.002) compared to *Vibrio cholerae* treated AD-MSCs and 14.76±5.79 fold (p=0.01) was increased compared to *Vibrio cholerae* treated Caco-2 cells.

The gene expression of the *IL-8* in exposure to *Vibrio cholerae* increased by 31.47±3.99 fold in AD-MSCs ($p<0.0001$), 3.1±0.43 fold in Caco-2 cells (p=0.79), and 16.1±3.21 fold in the co-cultured group (p=0.001). The *IL-8* expression in the *Vibrio cholerae* treated co-cultured group was decreased by 3.34±0.43 fold (p=0.27) compared to *Vibrio cholerae* treated AD-MSCs and 6.18±1.38 fold (p=0.17) were increased compared to *Vibrio cholerae* treated Caco-2 cells.

Evaluation of Apoptosis

As shown in Figure 6, the mean percentage of apoptosis of AD-MSCs was % 4.5±3.66 and in exposure to 5×10^6 CFU *Vibrio cholerae* was reached 59.22%±2.04 ($p<0.0001$). The mean percentage of apoptosis of Caco-2 cells was 3.1%±1.63, which was reached 45.58%±1.16 in exposure to *Vibrio cholerae* ($p<0.0001$). The mean percentage of apoptosis of the co-culture group was also 1.17%±1.03, which was reached to 59.15%±2.05 in exposure to *Vibrio cholerae* ($p<0.0001$). The mean percentage of apoptosis in the *Vibrio cholerae* treated co-culture group was significantly increased compared to the *Vibrio cholerae* treated Caco-2 cells ($p<0.0001$).

Table 3. The Fold Regulation of *Ctx-B*, *TNF-a*, *interleukin (IL)-1B*, and *IL-8* gene expression in exposure to *Vibrio cholerae* in experimental groups.

	MSC+ Vibrio / MSC	Caco2+ Vibrio/Caco2	MSC*Caco2+Vibrio/MSC*Caco2	MSC*Caco2+Vibrio/ MSC +vibrio	MSC*Caco2+Vibrio/Caco2+vibrio
Ctx-B	1.15±0.59	3.06±0.57	1.4±0.12	-2.15±0.27	-3.57±0.94
TNF-α	33.94±0.16	139.6±5.47	31.89±0.77	1.8±0.04	6.39±0.9
IL-1β	13.02±1.14	4.76±1.51	19.1±0.43	-17.76±3.13	14.76±5.57
IL-8	31.47±3.99	3.1±0.43	16.1±3.21	-3.34±0.43	6.18±1.3

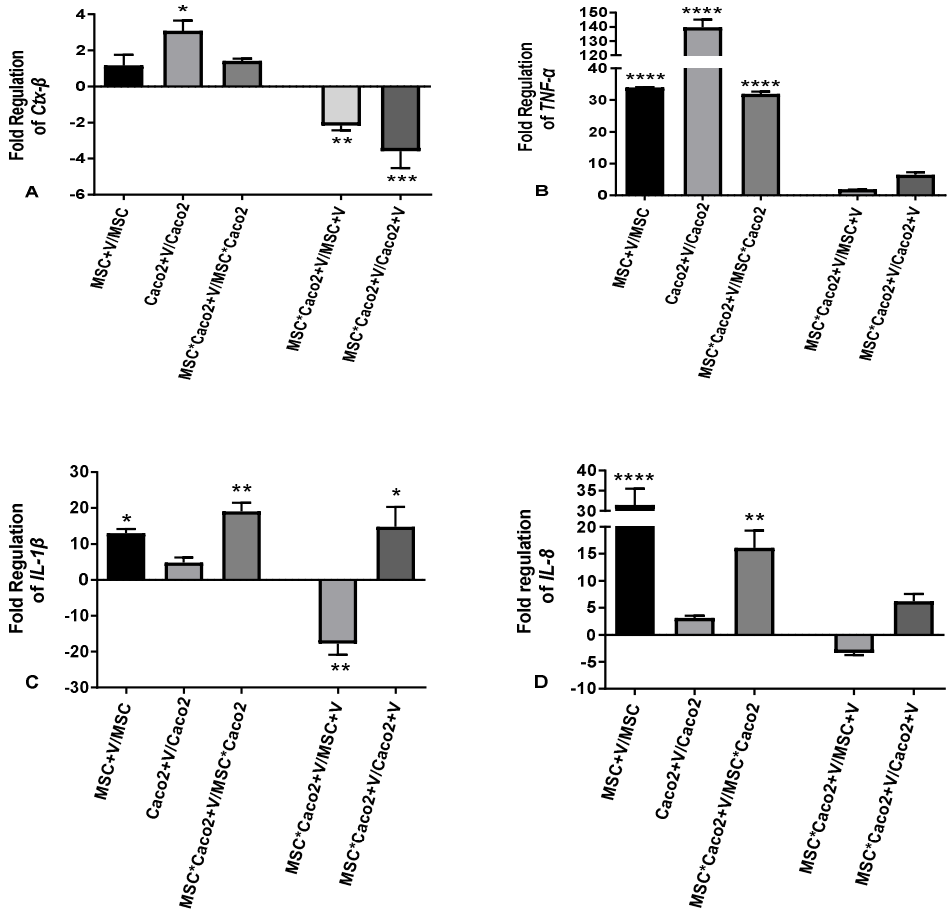

Figure 5. The Fold Regulation of *Ctx-B* (A), *TNF-a* (B), *interleukin (IL)-1β* (C), and *IL-8* (D) gene expression in the experimental groups compared to the control groups. In each bar chart MSC+V/MSC, Caco-2+V/Caco-2 and MSC*Caco-2+V/ MSC*Caco-2 shows the ratio of gene expression in the *Vibrio cholerae* treated to non-treated groups. In each bar chart, MSC*Caco-2+V/ MSC+V and MSC*Caco-2+V/ Caco-2+V show the ratio of gene expression in the co-culture groups to single-cell culture. *p<0.05, **p<0.01,*p<0.001 and ****p<0.0001**

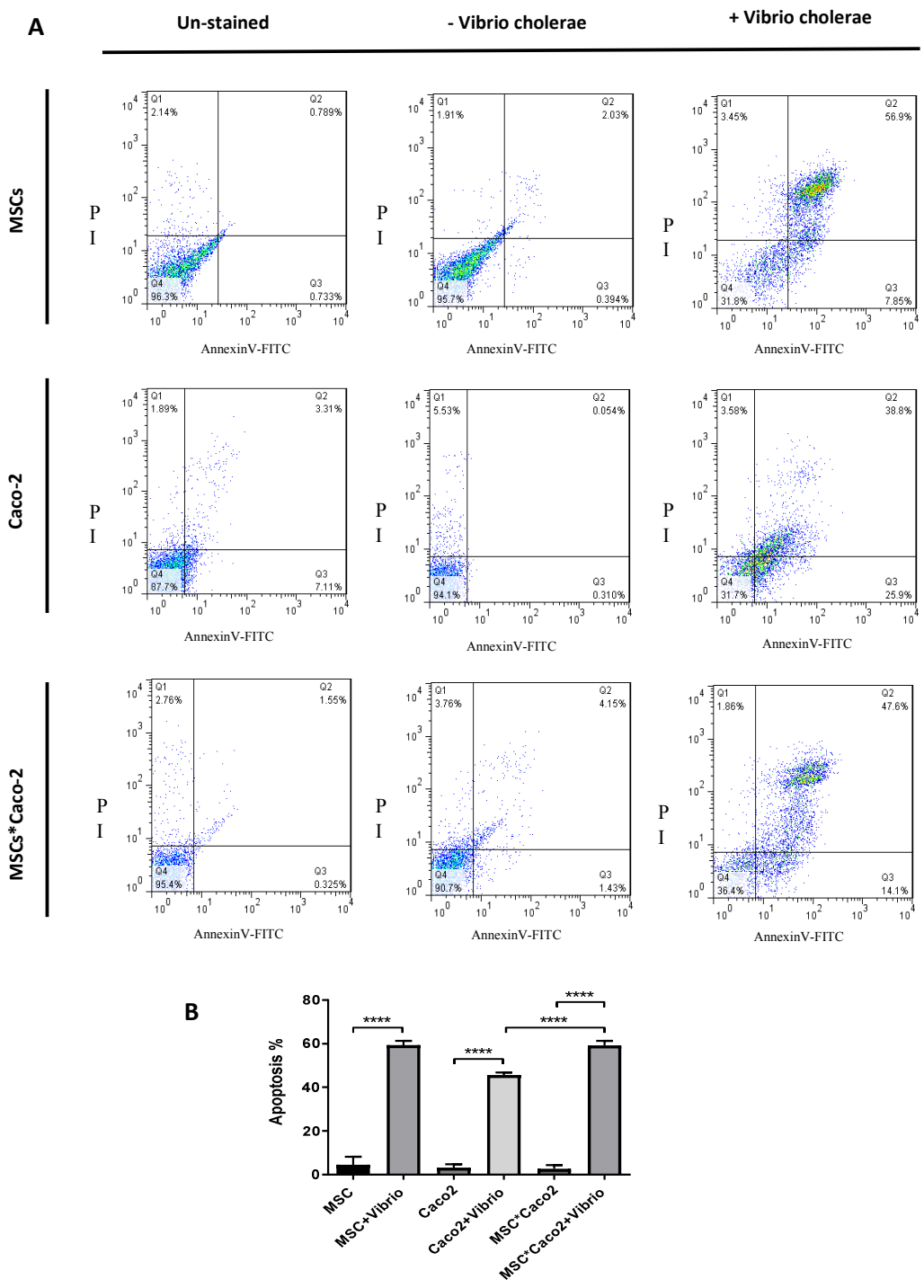

Figure 6. Evaluation of the effect of MSCs*Caco-2 co-culture on the rate of apoptosis in response to Vibrio cholerae infectionby annexin V staining. (A) Dot plot diagrams show the percent of necrotic cells (Q1), late apoptotic cells (Q2), early apoptoticcells (Q3), and viable cells (Q4) of un-stained or annexin (FITC) -PI stained groups, including MSCs, Caco-2 cells andMSCs*Caco-2 co-culture in the presence and absence of Vibrio cholerae. (B) The bar chart shows the mean percentage ofapoptosis±S.D. in the experimental groups. ** indicates that the two groups are statistically significant ($p \leq 0.0001$).**

DISCUSSION

The intestinal epithelium regulates the absorption of nutrients, water, and electrolytes and is the first defense barrier against intestinal toxins and pathogens. Epithelial cells are tightly connected and provide a strong barrier against the penetration of microorganisms, antigens, and toxins. *Vibrio cholerae* attaches to epithelial cells and leads to the production and secretion of chloratoxin. Chloratoxin in a positive feedback loop leads to the secretion of inflammatory cytokines, weakening of intercellular connections and destruction of the epithelial defense barrier, severe fluid excretion, and exacerbation of inflammation.[17] The present study was performed for the first time to investigate the immunomodulatory effects of MSCs on Caco-2 epithelial cell line in exposure to *Vibrio cholerae*. To achieve this goal, AD-MSCs were co-cultured with Caco-2 cells in the same wells, so that the Caco-2 cells were affected by both regulatory factors resulting from cell contact and secretions of AD-MSCs. Because the size of the AD-MSCs (17.9 to 30.4 μm) and Caco-2 cells (6 μm) are different, both of them occupy the same surface area 150,000 Coco-2 cells and 40,000 AD-MSCs are co-cultured in each well.

The attachment to the epithelial cells is the first step of *Vibrio cholerae* pathological effects. Reducing bacterial attachment to the cells will play a vital role in the production of chloratoxin and its destructive effects. For doing the present study, it was necessary to determine the number of bacteria that could cause the maximum Caco-2 infection while providing the time required to evaluate the effect of MSCs on the cellular secretions (NO, PGE2, IL-6). Therefore, in a preliminary study, Caco-2 cells were infected with different amounts of bacteria ranged from 5×10^3 to 5×10^7. According to the results, the higher the dose of bacteria terminated to the higher the rate of attachment and internalization to the cells, but at the same time, the rate of necrosis and cell destruction increased. The dose of 5×10^6 provides the maximum infection rate that could keep cells alive for up to 48 hours after infection for monitoring cell secretions. Therefore, the results of both 5×10^6 and 2×10^7 CFU were reported for attachment and internalization assay, but only 5×10^6 CFU was used for other experiments. The results showed that the bacterial attachment in the co-culture group of MSC and Caco-2 cells was significantly reduced compared to the Caco-2 cells. Decreased bacterial attachment in the co-culture group is probably due to the following reasons**:**

1) Antimicrobial peptides secreted by MSCs i.e. the human cathelicidin (hCAP-18/LL-37), β-defensin-2, lipocalin 2, and hepcidin.[1,18] 2) Indoleamine 2, 3-dioxygenase (IDO) secretion by MSCs prevents the formation of bacterial biofilms by indole degradation. Biofilm formation is one of the critical mechanisms in *Vibrio cholerae* pathogenesis. According to previous studies of the present group, MSC secretome significantly inhibits the formation of *Vibrio cholerae* biofilm.[1,18] IDO production also inhibits bacterial growth by tryptophan degradation.[19]

3) The MSCs secrete tissue inhibitors of metalloproteinase (TIMP). TIMP prevents the degradation of the extracellular matrix and the breakdown of tight junction proteins, leading to inhibition in bacterial attachment and penetration to the lower layers.[20]

The chloratoxin production and activation following the bacterial attachment is the main mechanism of intestinal epithelial cells destruction and clinical symptoms such as diarrhea. In the present study, the *Ctx-B* expression of Caco-2 cells as the main host of *Vibrio cholerae* was higher than the other groups. Co-culture of Caco-2 cells and MSCs reduced chloratoxin expression compared to Caco-2 cells in exposure to *Vibrio cholerae*. To the best of our knowledge, no study has been conducted on the effectiveness of MSCs on the production of bacterial toxins; But it appears that decreased bacterial attachment is the major reason for the reduction in *Ctx-B* expression. Numerous studies have shown the preventive role of MSCs in destructive and pro-inflammatory effects of toxins such as staphylococcal enterotoxins and endotoxin of acute respiratory infections causing bacteria.[21] Decreased bacterial attachment and chloratoxin expression have a positive effect on epithelial barrier integrity.

IL-6 plays a key role in the protection of intestinal mucosa integrity. Studies have shown that IL-6 reduces the rate of apoptosis by expression of *Bcl-xL*, *Mcl-1*, *cIAP-2*, and *Bcl-3* anti-apoptotic proteins in exposure to *Citrobacter rodentium*. The study of Kuhn et al showed that IL-6 is involved in the proliferation of epithelial cells and repairing the epithelial barrier.[22] Inhibition of IL-6 in patients with rheumatoid arthritis

improved the disease, but cause severe epithelial barrier damage.[23] In this study, both MSCs and Caco-2 cells produced significant amounts of IL-6 that were reduced in exposure to *Vibrio cholerae*. Previous studies have shown that *Vibrio cholerae* infection causes an increase in IL-6 in the gut,[2] but this increase depends on the presence of innate immune cells such as PMNs.[24] It seems that in the absence of the help of innate immune cells, *Vibrio cholerae* has an inhibitory effect on the IL-6 production by MSCs and Caco-2 cells. In this regard, the production of IL-6 is enhanced in the MSCs-Caco-2 co-culture groups and secreted higher levels of the IL-6 in response to *Vibrio cholerae* infection.

Prostaglandins are synthesized from arachidonic acids by cyclooxygenases and play an important role in inducing inflammation and pathogenesis of chronic inflammatory diseases.[25] Studies have shown that chloratoxin increases cAMP which leads to phospholipase C activation, hydrolysis of membrane phospholipids, accumulation of arachidonic acid, and PGE2.[26] PGE2 and PGF2 recruit the inflammatory cells (neutrophils, mast cells, macrophages, and other immune cells), exacerbate the inflammatory responses, and destruct the epithelial barrier.[27] Both Caco-2 and MSCs produce PGE2 at a steady state. The PGE2 production was decreased in AD-MSCs and slightly increased in Caco-2 cells in exposure to *Vibrio cholerae*. PGE2 production from MSCs is IL-6 dependent.[28] MSCs secrete PGE2 and IDO in the presence of IL-6 and induces M2 macrophages polarization, suppress inflammatory responses, and increase tissue repair. Whereas, MSCs do not produce PGE2 in the absence of IL-6, and direct macrophages to the inflammatory M1 phenotype. The effect of interleukin-6 on PGE2 production explains why prostaglandins decrease in *Vibrio cholerae* infected cells.

Despite the lower bacterial attachment to MSCs compared to Caco2 cells, the rate of bacterial internalization to MSCs is higher than that of Caco-2 cells. Bacterial internalization to intestinal epithelium does not appear to be important in the pathogenesis of *Vibrio cholerae*, but it is important to evaluate the effects of bacterial internalization on MSCs and subsequent inflammatory responses.[29] In the present study, the bacterial internalization to the co-culture group of MSCs and Caco-2 epithelial cells significantly increased compared to Caco-2 epithelial cells. While the rate of bacterial attachment in the co-culture group was reduced compared to Caco-2 cells, the probable reason is the high expression of *matrix metalloproteinases (MMP)* on MSCs along with the restriction factors of bacterial attachment that were described earlier. MMP in pathological conditions and the presence of bacteria causes extracellular matrix digestion, exposure of cell membranes, and facilitates bacterial internalization to the cells.[30] MSCs produce high levels of TIMP, IDO, lipocalin, and other antimicrobial compounds in their secretions in addition to the extraordinary level of surface MMP. Therefore, the rate of bacterial attachment and internalization in the co-culture group respectively was decreased and increased in exposure to *Vibrio cholerae*.

Bacteria in both membrane-bound and intracellular forms can activate the signaling pathways of inflammatory cytokine production through its virulence factors.[31] With increasing bacterial attachment and invasion, various inflammatory signaling pathways such as NF-kB and MAPK may be initiated and leading to intensification of inflammatory responses and proinflammatory cytokine (*TNF-α*, *IL-1B*, and *IL-8*) expression.[32] In the present study, the expression of *IL-1B*, *IL-8*, and *TNF-α* was increased after exposure to *Vibrio cholerae* in all experimental groups post-exposure to *Vibrio cholerae*. However, the expression of *IL-1β* and *IL-8* in the co-culture group was lower than that of MSCs and higher than the Caco-2 epithelial cells in exposure to *Vibrio cholerae*. Based on the obtained data, it seems that inflammatory cytokine gene expression has a direct correlation with the rate of *Vibrio cholerae* internalization.

Numerous studies show that epithelial cells in exposure to *Vibrio cholerae* induce pro-inflammatory cytokines (such as TNF-α, IL-1B, and IL-8), inflammatory cell proliferation, and lead to progressive inflammatory responses.[24] These pro-inflammatory cytokines cause the degradation of tight junction proteins, cell apoptosis, and consequently increased antigen penetration to the underlying layers and exacerbation of inflammation.[33] In the present study, the rate of apoptosis in the co-culture group was significantly higher than the Caco-2 cells in exposure to *Vibrio cholerae*. The higher apoptosis of the co-culture group is probably related to the high expression of *MMP* on MSCs, more bacterial internalization, and consequently increased expression of pro-inflammatory cytokines. According to the study of Kiesslich et al, TNF-α leads to the formation of intercellular spaces and a 27-fold increase in cell loss and severe destruction of

the epithelial barrier.[34] In the study of Ma et al, treatment of Caco-2 cells with TNF-α reduced the expression of intercellular binding proteins.[35] According to the study of Al-Sadi et al, IL-1β increases the permeability of tight junction of Caco-2 cells in vitro and in vitro via P38 kinase activation, ATF-2 transcription factor, and increase in *MLCK* gene expression.[36]

AD-MSCs decrease bacterial attachment and colonization by secretion of various antimicrobial peptides, IDO and TIMP. Decreased bacterial attachment reduced the expression of chloratoxin and increased the IL-6 secretion, which should have a positive effect on epithelial barrier integrity. But increased bacterial internalization to AD-MSCs stimulates the inflammatory responses. The gene expression of *TNF-α*, *IL-1β*, and *IL-8* pro-inflammatory cytokines was increased and apoptosis was induced by degradation of the tight junction. Therefore, AD-MSCs show dual effects on inflammatory response and epithelial barrier integrity by reduction of bacterial attachment and increasing bacterial internalization. The probable reason for the dual effects is the high expression of surface matrix metalloproteinases on MSCs along with the high secretion of TIMP and other antibacterial peptides. It appears that the reduction of bacterial internalization is a suitable therapeutic approach in the limitation of inflammatory responses. Therefore, it is recommended that future studies focus on the protective effects of MSCs secretome.

CONFLICT OF INTEREST

The authors state that they have no conflict of interest in this study.

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