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Mesenchymal Stem Cell Therapy Mitigates Acute and Chronic Lung Damages of Sulfur Mustard Analog Exposure

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ABSTRACT

Sulfur mustard (SM) is an established chemical weapon that can result in severe damage to parts of the body. Currently, there are no effective treatments available for SM-caused damage. We aimed to investigate the therapeutic potential of adipose-derived mesenchymal stromal cells (AD-MSCs) and conditioned medium (CM-MSCs) in acute and chronic pulmonary mouse models caused by 2-chloroethyl ethyl sulfide (CEES), an SM analog.

The mice were divided into 4 experimental groups:(1) CEES+AD-MSCs, (2) CEES+CM-MSCs, (3) CEES, and (4) control. The model observation time was divided into 7 days for the short and 6 months for the long term. AD-MSCs were injected into mice via intraperitoneal injection 24 hours after CEES exposure. The therapeutic effects of AD-MSCs on pulmonary tissue damage were assessed using a histopathologic assay, measuring the neutrophil count, and bronchial alveolar lavage fluid (BALF) protein level. The levels of inflammatory and anti-inflammatory cytokines were evaluated using the enzyme-linked immunosorbent assay as the outcomes of interest.

Lung damage progression was reduced by AD-MSC treatment in mice after CEES injection into the peritoneum. The proportion of $CD11b^{+}F4/80^{+}$ macrophages in the peritoneum was significantly lowered by AD-MSC treatment following CEES exposure. AD-MSC administration also reduced the level of pro-inflammatory cytokines, BALF protein, and nitric oxide levels in the peritoneal cavity.

By reducing inflammation and enhancing tissue healing, AD-MSCs and CM-MSC help prevent acute lung damage caused by CEES. The current study supports the use of a mouse model as a solid experimental foundation and indicates potential use for future cell treatment.

Keywords: Bronchoalveolar lavage fluid; Conditioned medium; Mesenchymal stem cell; Peritoneal macrophage; Sulfur mustard

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INTRODUCTION

Sulfur mustard (SM) is a highly toxic biofunctional mustard agent and a chemical weapon that is notorious for causing severe and lasting injuries to different parts of the body. Notably, the lungs, eyes, skin, and mucous membranes are especially vulnerable to its devastating effects.[1] The use of SM in warfare and terrorist activities is a serious danger, and finding effective ways to prevent and treat the acute and chronic pulmonary complications it causes is a crucial challenge.[2,3] Exposure to SM induces immediate and delayed damage to various organs, with lung injury being the major cause of death.[4-10] Although some aspects of the SM action mechanism are known, more research is needed for complete elucidation.

In previous studies, it was found that adipose-derived mesenchymal stromal cells (AD-MSCs) reduced the production of pro-inflammatory cytokines, although there was no statistically significant impact on anti-inflammatory cytokines.[10] This lack of successful therapy for SM exposure highlights the urgent need for effective interventions upon the onset of acute symptoms to prevent long-term complications. The rapid and nonspecific effects of SM make current treatments inadequate for affected patients, as they often experience airway damage that requires specialized resuscitation methods.[11,12] This emphasizes the importance of timely interventions to mitigate the effects of SM.

MSCs are a type of stem cells that do not produce blood cells and can renew themselves. They are obtained from various sources such as the liver, placenta, fat, and skin.[13] The wide range of effects that MSCs have on the body, such as maintaining tissue balance, promoting regeneration,[14,15] and modulating the immune system, make them attractive candidates for therapeutic applications.[16] Studies indicate that the interaction between alveolar macrophages (AMs) and MSCs plays a key role in the therapeutic benefits of MSCs.[17,18] MSCs are also capable of converting macrophage into a less inflammatory and more anti-inflammatory type.[19,20] Furthermore, the conditioned medium (CM) obtained from MSCs (CM-MSC) contains a variety of cytokines and soluble factors that mimic the effects of MSCs in healing tissues, reducing inflammation, and modulating immunity.[21]

Human studies face ethical challenges and limited sample sizes, hindering progress. Utilizing animal models can address these issues and explore how SM impacts them. Unlike previous studies that focused solely on acute models and intravenous bone (B)-MSC treatment, this study examines the use of AD-MSCs for intraperitoneal administration in both acute and chronic models, which is an innovative aspect of the work. Our study aims to assess the immunomodulatory effects of MSCs in reducing complications arising from short- and long-term pulmonary and peritoneal inflammation in an animal model exposed to 2-chloroethyl ethyl sulfide (CEES). To the best of our knowledge, this innovative approach represents the first exploration of MSCs' potential to prevent complications from both short- and long-term pulmonary and peritoneal inflammation in a CEES-exposed animal model.

MATERIALS AND METHODS

Preparation of Experimental Animals and Treatments

Male C57/BL6 mice aged 6 to 8 weeks were purchased from the Royan Institute of Iran. They were kept in standard conditions with unlimited access to food and water, following a 12-hour light-dark cycle, at a constant temperature of 23±1°C, and 55%±5% humidity. The Animal Care Committee of Shahid Beheshti University approved all animal procedures (protocol number IR.SBMU.MSP.REC. 1399.339).

The mice were divided into 4 experimental groups: (1) CEES+AD-MSCs, (2) CEES+MSC-derived CM, (3) CEES, and (4) control. The animals were then categorized into acute (7 days) and chronic (6 months) groups, following a similar method used in previous research. The doses of CEES were determined based on weight and administered through intraperitoneal (IP) injection of 100 µL herbal sesame oil containing 10 mg/kg CEES. The model was validated using clinical and histopathological tests, as per the methods described in past studies. The control group received 100 µL of solvent oil (IP). After either 7 days (acute) or 6 months (chronic), 3 mice from each group were euthanized with sodium pentobarbital (Sigma Aldrich, Louis, USA, 120 mg/kg). Organs, like lungs, were collected for histopathological or tissue change analysis.[22]

Twenty-four hours after CEES model induction, mice received either 500 µL of AD-MSCs (1×10^6 cells/mL) or phosphate buffer solution (PBS)(control), or 200 of µL CM-MSC (IP) twice a week for 1 week. After either 1 week (acute) or 6 months (chronic), the

animals were humanely euthanized, and their lungs were isolated for histological evaluation. Additionally, samples of bronchoalveolar lavage fluid (BALF), serum, and spleen cell culture supernatants were collected for cytokine analysis.

Isolation and Culture of Murine AD-MSCs

After spinal cord transection in mice, the abdominal fat was meticulously separated. It was then placed under the influence of type I collagenase 0.1%, at 37°C for 30 minutes. The fat cells were then transferred to a culture medium after 20 minutes of centrifugation at 450*g*. After 24 hours, the adherent cells were washed 3 times, and fresh culture medium was added.[21,23]

Osteogenic and Adipogenic Differentiation Potential of MSCs

When the cell cultures reached 80% confluency, to evaluate the ability of MSCs in differentiating osteoblasts and adipocytes,[10] we replaced the medium of the second passage of MSCs with a specialized complete differentiation medium of osteogenic and adipogenic. After 21 days, the accumulation of intracellular calcium and lipids was detected by Alizarin Red-S, and Oil Red O, respectively.[21]

Immunophenotyping MSCs

The flow cytometry technique was employed to detect surface markers related to the AD-MSCs. Passage 2 AD-MSCs were labeled with monoclonal antibodies targeting mouse CD105, CD34, CD45, CD11b, CD73, and CD90. Staining followed the manufacturer's instructions.

Preparation of CM-MSCs

The medium was substituted with less FBS every 2 to 3 days in order to adjust cells to the serum-free medium. The supernatant was clarified by passing it through a 0.22-μm filter to eliminate any debris, and it was subsequently preserved at −70°C as CM-MSC.

Preparation of Peritoneal Lavage and Isolation of Peritoneal Macrophages in Acute Group

Mice were euthanized with pentobarbital sodium until unresponsive to a toe pinch, and then 5 to 7 mL of ice-cold PBS was administered through the peritoneal membrane under the abdominal muscles.[24] Gentle massage ensured complete coverage by cold RPMI 1640 medium, which was then aspirated.[25] Peritoneal cells were washed with 5 mL of RPMI 1640 (Invitrogen, Darmstadt, Germany) and centrifuged at 200*g* for 10 minutes. After washing with PBS, cells were cultured in RPMI 1640 Complete medium at 37°C for 4 hours.[26] Adherent peritoneal macrophages (PEMs) were used for further MTT assays.

Bronchoalveolar Lavage Fluid Collection

In brief, the mice were deeply anesthetized, and their chest was opened to allow for left ventricular heart puncture, which was performed using a syringe that had been treated with heparin. A tracheal cannulation was then carried out, and 1 mL of Dulbecco's Modified Eagle Medium (DMEM) culture was used to perform lavage 5 times.[27,28] The BALF underwent centrifugation, and the resulting supernatants were preserved at −80°C for subsequent cytokine examination.

Flow Cytometry Analysis of PEM in Acute Model

Peritoneal lavage was harvested as described above. After blocking nonspecific binding with an FcR blocker, 10^6 peritoneal cells were suspended in 1 mL washing buffer containing 0.5% bovine serum albumin (BSA) in PBS. These cells were then stained with CD11b-PE/Cy7 (clone M1/70) and F4/80-PE (clone BM8) antibodies at 4°C for 20 minutes to evaluate surface markers. Gating was performed using FlowJo-v10 software, first defining the macrophage cell population based on forward scatter (FSC) vs. side scatter (SSC). Peritoneal macrophages were identified by co-expression of F4/80 and CD11b.

BALF Protein Concentration

The procedure involved the following steps: a) BALF was centrifuged immediately for 15 minutes at 1500 rpm; b) the supernatant was removed and stored in sterile Eppendorf tubes at −80°C c) total protein was measured using a BCA protein assay kit Bio Basic, with bovine serum albumin was used as the standard. A microplate reader was used to analyze the provided samples at 562 nm.[35] Then, 25 μL of the provided sample along with the standards as well as 200 μL of the solution were added to the container (a tube), homogenized, placed for an hour in a bain-marie at 60°C, and then read at a wavelength of 570 to 620 and the amount of protein was calculated.

Measurement of Cytokines (Outcome Variables)

Measuring the levels of various cytokines of tumor necrosis factor (TNF)-α, interleukin (IL)-17, interferon

(IFN)-γ (as pro-inflammatory markers), and IL-10 (as anti-inflammatory marker) in spleen lymphocyte and peritoneal culture supernatants, serum, and BALF was done using enzyme-linked immunosorbent assay (ELISA) kits provided by R&D Systems (UK). These cytokines were considered the primary outcomes of interest in the present study.

Lung Tissue Histological Analysis

Mice were deeply anesthetized with pentobarbital sodium until unresponsive to toe pinch. The inflation of the lungs was established with 1 mL of 10% paraformaldehyde via a tracheal catheter and it was fixed in 10% formalin. Several sections of size 5 millimeters were stained by using hematoxylin, and eosin (H&E). Then, the inflammatory cell infiltration, goblet cell hyperplasia, and collagen fibers were assessed. A blinded pathologist analyzed histopathological changes, capturing images with a Labomed Lx500 light microscope.

Nitric Oxide Test

Nitric oxide (NO) amounts in PEMs and spleen lymphocyte culture medium were measured using the standard Griess reagent. A microplate reader was used to read the absorbance at 540 to determine NO levels in the acute model as the secondary outcome of interest.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

PEM and splenic lymphocytes were cultured at 1×10^6 cells/mL. A sterile MTT reagent (Sigma, 5 mg/mL final concentration in PBS) was added to each well. After incubation at 37°C for 4 hours, wells were washed. Then, the formazan crystals were dissolved by adding DMSO (Sigma, 100 µL). Measuring the absorbance rates was implemented at 570 nm.[29]

Bronchoalveolar Lavage Fluid Neutrophil Counts

For BALF neutrophil measurement, cells were collected by centrifugation (400*g*, for 10 minutes), the pellet was resuspended in PBS with 1% BSA, and the total cells were counted with a Neubauer chamber. Neutrophil counts were also considered as the secondary outcome of interest.

Statistical Analysis

The quantitative variables like various cytokine levels were presented using mean±SD. Analysis of variance (ANOVA) technique was used to compare flow cytometry results and cytokine concentrations across groups. Moreover, pairwise comparisons were conducted using the Tukey method. Comparative groups included 1) CEES-untreated, 2) CEES+AD-MSC, 3) CEES+CM-MSC, and 4) control. The normality assumption for the variables was assessed using the Kolmogorov-Smirnov test. p values≤0.05 were considered statistically significant. The data were analyzed in IBM-SPSS version 23.0 (IBM Corp., Armonk, NY).

RESULTS

Characterization of MSC from Adipose Tissue

Flow cytometry analysis showed the expression of specific cell surface antigens on AD-MSCs: CD73 (89%), CD90 (92.5%), and CD105 (91%). They did not express CD11b (1.17%), CD34 (1.8%), and CD45 (1.9%) (see Figure 1A). Figure 1B shows a microscopic view of AD-MSC morphology. Furthermore, AD-MSCs were able to differentiate into adipogenic and osteogenic lineages, as evidenced by morphological changes and accumulation of lipid droplets (Oil Red O staining) in differentiated cells, and mineralization of the extracellular matrix after 21 days (see Figures 1C and 1D).

Histopathological Analysis of Lung Tissue

Figure 2 (A–F) shows examples of pathological changes induced by CEES in the model and treatment groups CM-MSC and AD-MSC for acute and chronic conditions The untreated acute model displayed thickening of alveolar walls, infiltration of mononuclear cells, hyperplasia of smooth muscles around airways, fibrin strands around blood vessels, edema, chronic interstitial pneumonia, and cuboidal or epithelialized alveolar wall cells with remaining type 2 pneumocytes. Treatment with AD-MSC and CM-MSC significantly reduced the severity of these lesions, leading to a decrease in smooth muscle hyperplasia, mononuclear cell infiltration, and alveolar wall thickness (resulting in a squamous appearance and reduced thickness).

The untreated chronic group exhibited chronic interstitial pneumonia with characteristics such as mononuclear cell infiltration, interstitial fibrosis, and smooth muscle hyperplasia in the bronchial wall. There were also observations of smooth muscle hyperplasia and remaining type 1 pneumocytes around blood vessels and airways (arteriosclerosis), indicating the presence of interstitial fibrosis. On the other hand, chronic models

treated with AD-MSC or CM-MSC showed a significant decrease in histological evidence of chronic interstitial pneumonia, with minimal inflammatory cell infiltration and smooth muscle hyperplasia near airways and vessels. However, reactive nuclei (vesicular and hypochromatic) were still visible (see Figure 2).

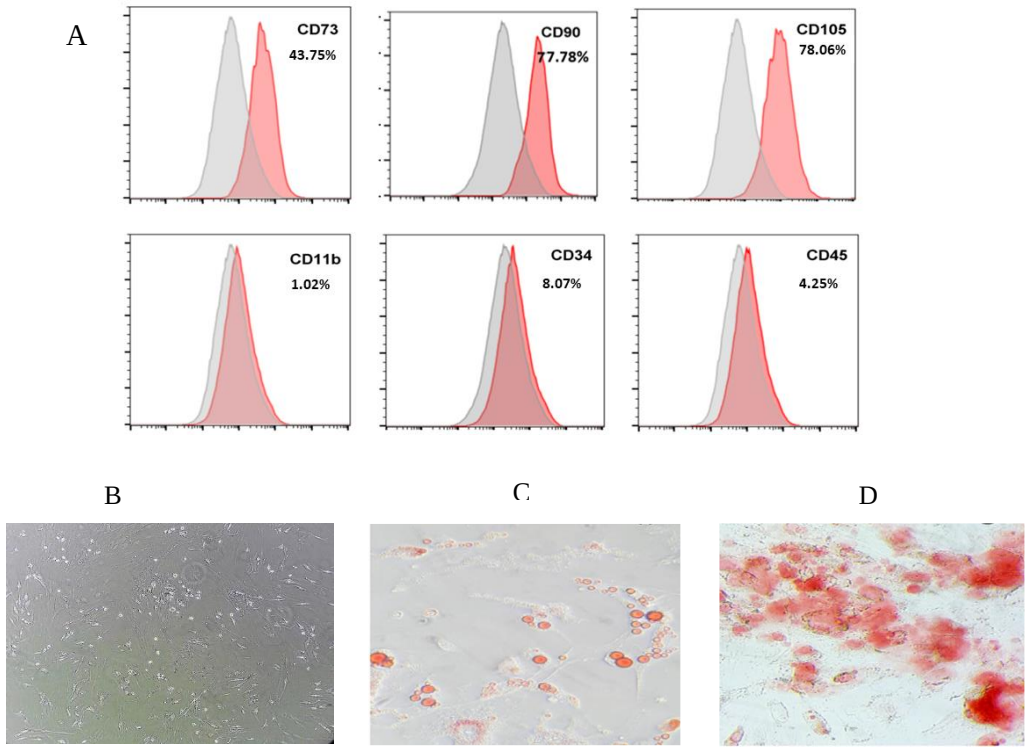


Figure 1. Characterization of adipose-mesenchymal stem cell (AD-MSC) isolated from adipose tissue. A. Evaluation of AD-MSC phenotype by flow cytometry; B. microscopic view of AD-MSC morphology; C. Differentiation potential of MSCs to adipogenic, droplets of fat in the cell are characterized by Oil Red O staining; D. Differentiation potential of MSCs to osteogenic, calcium-containing precipitates are characterized by Alizarin Red S staining.

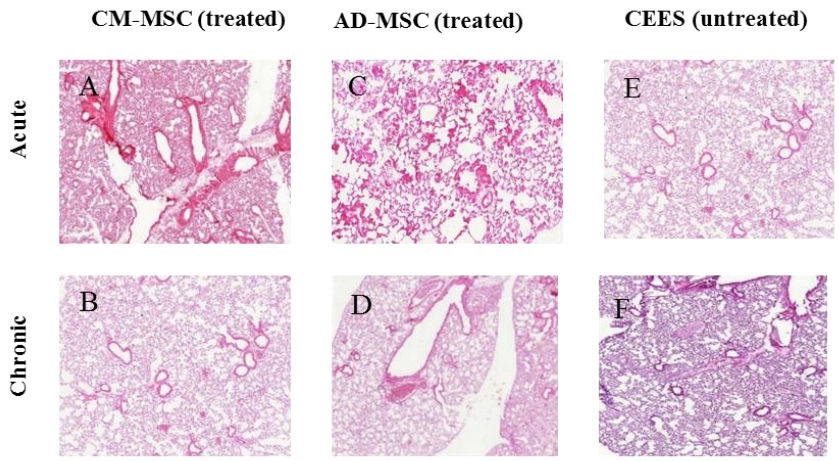


Figure 2. Representative images of the lung from experimental animals. Morphological deviations in 2-chloroethyl ethyl sulfide (CEES) (untreated) acute (A), CEES (untreated) chronic (B), adipose-derived mesenchymal stromal cells (AD-MSC) (treated) acute (C), AD-MSC (treated) chronic (D), conditioned medium (CM-MSC) (treated) acute (E), CM-MSC (treated) chronic (F).

Cytokine Level Assessment

Acute Model

ELISA was used to measure pro-inflammatory and anti-inflammatory cytokines in BALF, spleen lymphocytes, and serum of acute model mice. It was found that exposure to CEES significantly increased levels of TNF-α, IL-17, IFN-γ, and IL-10 compared to the control group (see Figure 3, red bars (CEE+)).

Treatment with CM-MSC increased the serum level of anti-inflammatory IL-10 compared to the CEES group (Figure 3, blue bars (CM+)). However, IL-10 levels in BALF and the spleen decreased slightly. Similarly, treatment with AD-MSC increased IL-10 in the spleen compared to the CEES group (see Figure 3, green bars (ADMSC+)), but not statistically significant. Levels of IL-10 in the serum and BALF showed a slight nonsignificant decrease after treatment with AD-MSC. Both AD-MSC and CM-MSC treatment effectively suppressed the expression of pro-inflammatory TNF-α, IL-17, and IFN-γ in the lungs and spleen lymphocyte supernatant (see Figure 3). It is important to note that there was a significant reduction in serum IFN-γ levels after treatment ($p<0.001$).

Chronic Model

Figure 4 compares cytokine levels (IL-10, IL-17, IFN-γ, and TNF-α) in serum, spleen, and BALF samples from the chronic model to assess the impact of CEES on their balance. Notably, the CEES group (untreated) displayed elevated expression of all 4 cytokines in all 3 tissue types. The results of the chronic model in this study showed that the IL-10 was significantly upregulated in CM-MSC and AD-MSC compared to the control in BALF ($p<0.01$). Conversely, IL-10 in the spleen and serum decreased with CM-MSC treatment compared to the untreated CEES group (tended to decrease slightly but did not show any statistical significance). It was also observed that the IL-17 level was significantly downregulated in CM-MSC ($p<0.05$) and the AD-MSC group ($p<0.05$) compared to the CEES+ in BALF and spleen and tended to decrease slightly but did not show any statistical significance in AD-MSC serum, as well as in CM-MSC ($p<0.05$) compared to the CEES+ in serum. The level of TNF-α was significantly lower in the groups treated with CM-MSC and AD-MSC compared to the group treated with CEES in the BALL and spleen ($p<0.01$ and $p<0.05$ respectively). There was a slight decrease in TNF-α level in serum after treatment, but it was not statistically significant. Following the administration of AD-MSC and CM-MSC, there was a further reduction in INF-γ expression in the lymphocyte supernatant of both BALF and spleen, although this was also not statistically significant in serum ($p<0.05$). After the administration of AD-MSC and CM-MSC, the expression of INF-γ was further reduced in both BALF and spleen lymphocyte supernatant but did not show any statistical significance in serum ($p<0.05$) (Figure 4).

Cytokine Assessment in Intraperitoneal Lavage of Acute Mouse Model

Figure 5 depicts the levels of 4 cytokines (IL-10, IL-17, INF-γ, and TNF-α) measured in the medium of PEMs in acute models to assess the effects of CEES on cytokine balance. Mice in the CEES group exhibited upregulated TNF-α ($p<0.01$), IL-10 ($p<0.01$), INF-γ ($p<0.05$), and IL-17 ($p<0.001$) expression. These cytokines were significantly downregulated in AD-MSC and CM-MSC compared to the CEES.

Lung Protein

BALF total protein is an important indicator of pulmonary edema exudation. CEES-treated mice exhibited elevated BALF protein levels compared to the control group. Treatment with CM-MSC and AD-MSC resulted in a reduction in the protein levels ($p<0.001$) (Supplementary Figure 1).

Nitric Oxide Test

Treatment with both CM-MSC and AD-MSC resulted in a significant reduction in the NO levels ($p<0.001$) (Supplementary Figure 2).

Cell Viability

Based on the findings, the CEES was shown to lower cell viability in spleen lymphocytes and PEMs compared to the control group. Interestingly, mice treated with CM-MSC and AD-MSC groups displayed a notable increase in lymphocyte viability ($p<0.001$) (Supplementary Figure 3).

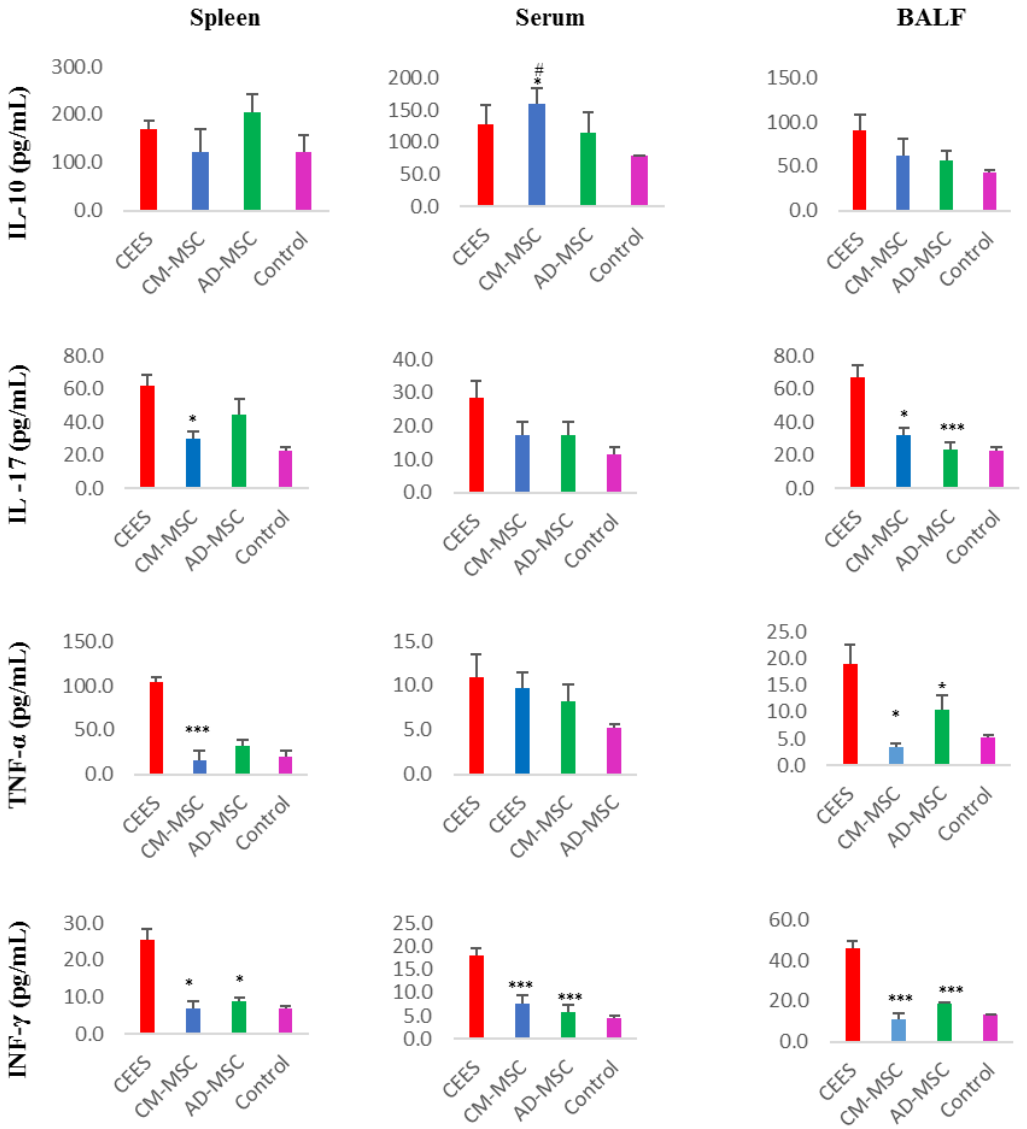


Figure *3*. Effects of intraperitoneal administration of conditioned medium (CM-MSC) and adipose-derived mesenchymal stromal cells (AD-MSC) on tumor necrosis factor (TNF)-α, interleukin (IL)-10, Interferon-gamma (INF-γ) and IL-17 expression in serum, spleen lymphocyte supernatant, and bronchial alveolar lavage fluid (BALF) in 2-Chloroethyl ethyl sulfide (CEES)-induced acute mouse model. *p* values of≤0.05 were considered statistically significant. **p*<0.05, ** *p*<0.01, and * *p*<0.001 treated vs untreated groups, # *p*<0.05, ## *p*<0.01 and ### *p*<0.001 AD-MSC+ and CM-MSC+ vs control.**

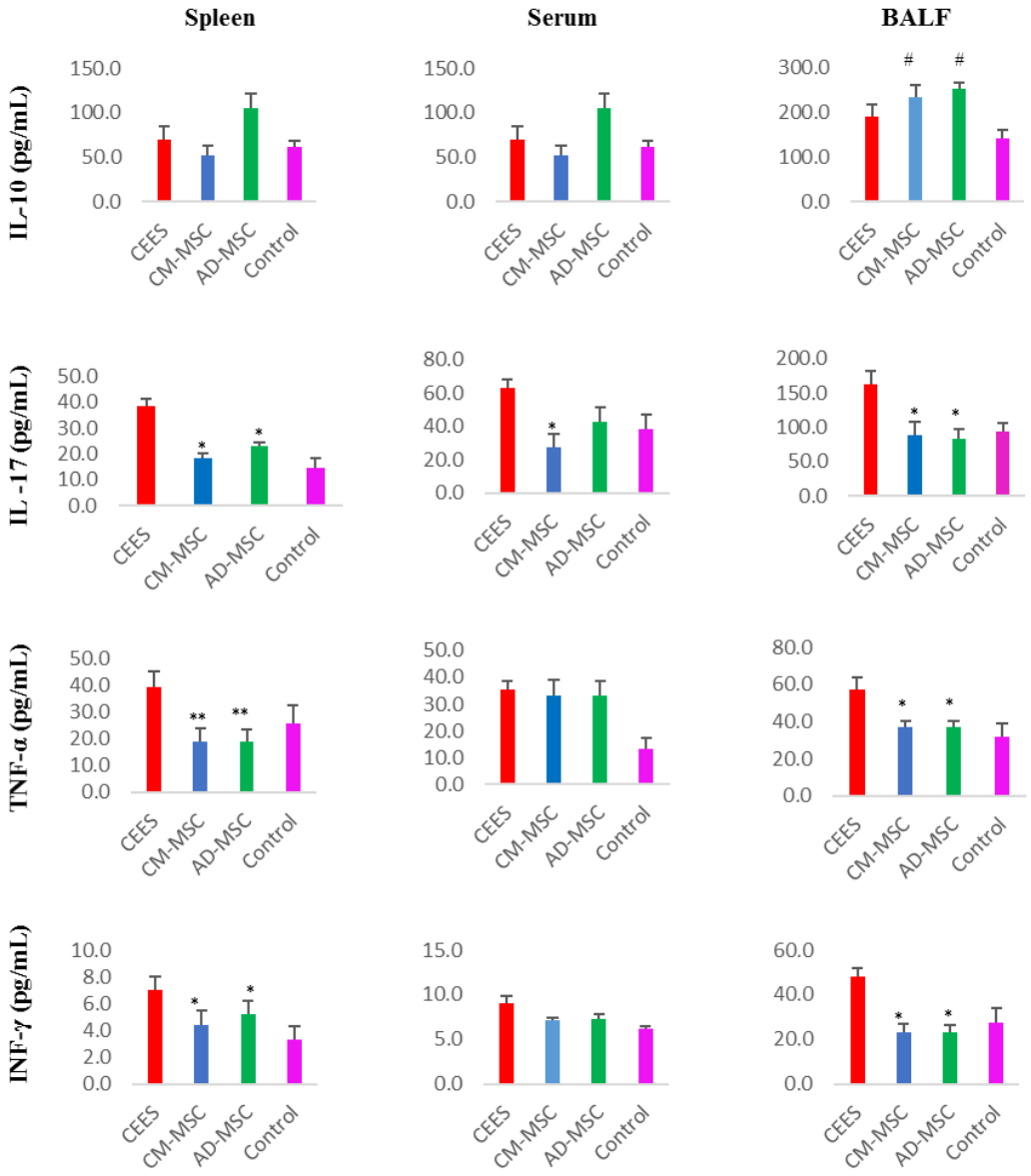


Figure 4. Effects of intraperitoneal administration of conditioned medium (CM-MSC) and adipose-derived mesenchymal stromal cells (AD-MSC) on tumor necrosis factor (TNF)-α, interleukin (IL)-10, interferon-gamma (INF-γ) and IL-17 expression in Serum, Spleen lymphocyte supernatant, and bronchial alveolar lavage fluid (BALF) in 2-chloroethyl ethyl sulfide (CEES)-induced chronic mouse model. $p \leq 0.05$ were considered statistically significant. *$p<0.05$, ** $p<0.01$, and *** $p<0.001$ treated vs. un-treated groups, $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ and $^{\#\#\#}$ $p<0.001$ AD-MSC+ and CM-MSC+ vs control.

Peritoneal Macrophages (PEMs)

Flow cytometry was performed to evaluate the effect of AD-MSC treatment on the macrophages in the peritoneum. PEMs are distinguished from other cells in the peritoneum based on high expression of CD11b and F4/80 (double positive). AD-MSCs and CM-MSC administration led to a considerable reduction in the frequency of peritoneal CD11b-positive and F4/80-positive macrophages (Figure 6; 11.1±1.4% and 13.1±1.6 respectively; $p<0.01$).

BALF Neutrophils

In the CEES-induced acute model, treatment with both CM-MSC and AD-MSC resulted in a significant reduction ($p<0.001$) in the number of neutrophils in BALF ($p<0.001$) (Supplementary Figure 4).

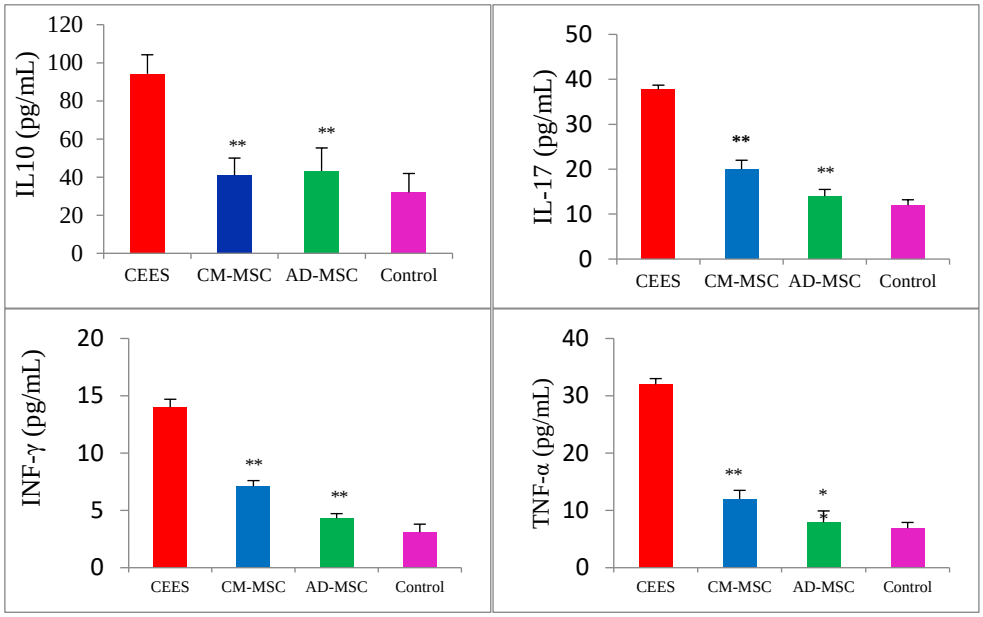
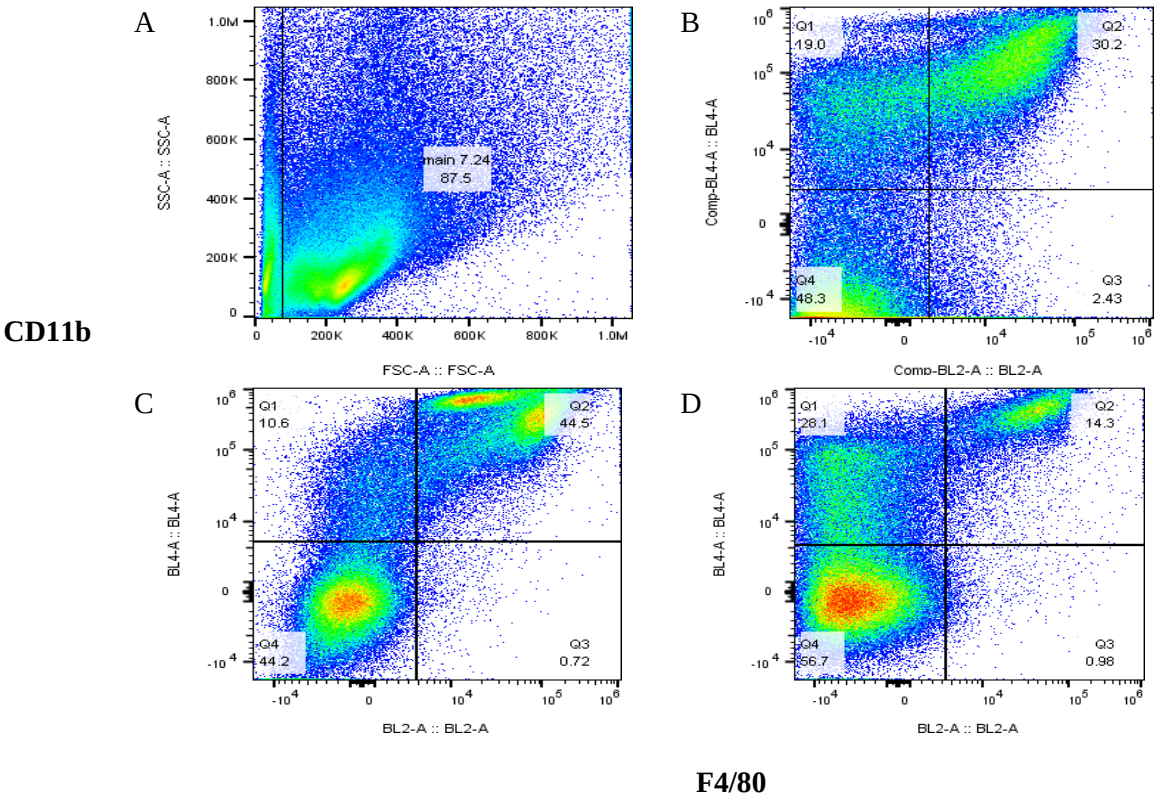


Figure 5. Effects of intraperitoneal administration of conditioned medium (CM-MSC) and sulfide (CEES)-induced acute mouse model. *p* values of ≤ 0.05 were adipose-derived mesenchymal stromal cells (AD-MSC) on tumor necrosis factor (TNF)-α, interleukin (IL)-10, interferon-gamma (INF-γ) and IL-17 expression in peritoneal macrophages (PEM) supernatant culture in 2-chloroethyl ethyl considered statistically significant. * *p*< 0.05, ** *p*< 0.01, and * *p*<0.001 treated vs untreated groups, # *p*< 0.05, ## *p*< 0.01 and ### *p*<0.001 AD-MSC+ and CM-MSC+ versus control group.**



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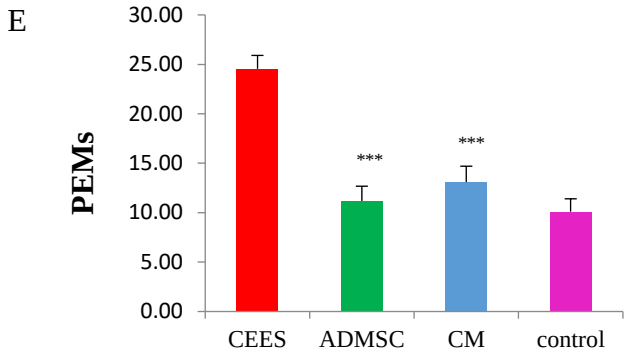

Figure 6. Flow cytometry analysis of peritoneal macrophages for phenotype examination. A. The gating strategy for peritoneal macrophage in the peritoneum. The main population was gated on a forward scatter area (FSC-A) vs. a side scatter area (SSC-A) dot plot. B. Peritoneal macrophages were differentiated based on the expression of CD11b and F4/80 C. untreated D. treated. E. Effects of intraperitoneal administration of conditioned medium (CM-MSC) and adipose-derived mesenchymal stromal cells (AD-MSC) on the percentage of CD11b+ and F4/80+ peritoneal macrophages in 2-chloroethyl ethyl sulfide (CEES)-induced acute mouse model.

DISCUSSION

This study used a specific model to understand both short-term and long-term complications from CEES exposure.[8,10] While recent research found that MSCs resist SM exposure and offer therapeutic benefits for SM-related injuries, the specific mechanisms behind their effectiveness, particularly in SM-induced acute lung injury, remain unclear.[8,10,11]

Animal studies have provided insights into the damaging effects of SM on the lungs. In the acute model, these studies have shown an accumulation of immune cells causing inflammation in the airways, along with an increased level of pro-inflammatory cytokines, and the presence of reactive oxygen species (ROS) and nitrogen compounds. Exposure to SM also leads to lasting changes in the immune system that contribute to the development of chronic lung fibrosis. This crucial finding deepens our understanding of the intricate mechanisms behind SM toxicity, particularly how it induces chronic inflammation and fibrosis in the respiratory system.[30,31]

After being exposed to SM, individuals may experience delayed toxicity to lung injuries. This is mainly caused by the harmful combination of oxidative stress and inflammation induced by ROS-induced oxidative stress. Administering MSC therapy shortly after exposure shows the potential to protect against these SM-induced lung injuries.[32,33] This experiment probed into what possible anti-inflammatory role of AD-MSCs in the treatment of lung injury is brought about by CEES. Several studies have demonstrated that CM-MSCs, which are endowed with numerous growth factors and functions in a way that is similar to MSC, could be employed as an alternative for treating diseases by means of tissue repair, in addition to having anti-inflammatory and immunomodulating properties.[10,21]

A previous study found that many inflammatory cytokines rise when there is SM.[8,10] Sadeghi and colleagues found that in the chronic mouse model, TNF-α declined significantly in the AD-MSC and CM-MSC group compared to that of the CEES-exposed. Similarly, Zhang and colleagues have shown that treating mice with AD-MSC decreases TNF-α and increases IL-10 concentrations in BALF.

On the other hand, Jung and colleagues showed that although the TNF-α levels declined slightly in BALF, there was no statistically significant difference between the lipopolysaccharide/ adipose-derived stem cells (LPS/ASC) and LPS groups. Our research investigated the anti-inflammatory effects of MSCs in both acute and chronic inflammatory models. We have observed that in AD-MSC or CM-MSC groups, there was a significant decrease in the levels of the pro-inflammatory cytokines, such as TNF-α, IL-17, and IFN-γ. This decrease has been identified in various tissues, such as BALF, splenic lymphocyte supernatant, and serum. The result affirms the anti-inflammatory potential of MSCs and highlights

their promising role in mitigating inflammation across different stages of the disease. On the contrary, in any of the groups, despite treatment with CM-MSC and AD-MSC, we did not observe any significant increase in IL-10 levels, which is known to participate in the immunomodulatory activity of stem cells and the differentiation of cells into immunomodulatory macrophages as we had expected.[43] This insignificant change was consistent with what was found in a previous study, in which there was no significant difference between the levels of IL-10 in the untreated group and those treated with MSCs and CM-MSCs in a chronic model.[10]

Injection of only one dose of stem cells in the acute disease model reduced the level of inflammatory cytokines. Probably, to maximize the stem cell's ability to induce IL-10 production, the injection should be done more frequently. Therefore, more studies should be done on how stem cells affect inflammation in general. If we want to fully understand this, it is important to determine the upregulation of anti-inflammatory cytokines, as well as the differentiation of macrophage cells and regulatory T cells, the latter of which play an important role in downregulating inflammation.

Recent research by Henao Agudelo et al. found that using extracellular microvesicles (MVs)-MSCs on a mouse reduced IFN-γ, IL-1β, and TNF-α levels in peritoneal lavage.[34] This finding aligns with our observations, where IL-17, IFN-γ, TNF-α, and IL-10 were broadly reduced in peritoneal lavage of mice treated with AD-MSCs.

According to what has been reported before,[35,36] decreased cell viability is a biological mechanism of CEES, and our study confirms this finding.

Severe ROS overproduction and the consequent oxidative stress are believed to be implicated in the SM damage. SM exposure can trigger various cellular and molecular pathways involved in generating ROS that eventually lead to cell death. In the acute phase, the production of ROS along with oxidative stress is increased by mechanisms such as diminishing antioxidant activity, depleting intracellular glutathione (GSH), reducing the efficacy of antioxidants that utilize GSH for their function, impairing mitochondrial function, promoting the recruitment leukocytes, and boosting inflammatory cytokines. Instead in the chronic phase, the upregulation of enzymes related to ROS production and the downregulation of antioxidant enzymes are the primary mechanisms by which SM results in oxidative stress. Therefore, therapy with potent antioxidants such as MSCs holds promise in mitigating the oxidative stress-related damage caused by SM.[37]

Neutrophils are considered the first immune cells flowing to the site of injury or inflammation.[38] Their accumulation in the airspaces is induced by cytokines and chemokines. Neutrophils are considered a key component in lung injury with either direct or indirect influences.[39] In the present study, lung neutrophils, cytokine, and protein levels in BALF were measured. Increased protein may be indicative of a compromised alveolar-capillary barrier.[39] Our results discovered that SM-exposed mice that received AD-MSC and CM-MSC had a reduction in lung edema and infiltration of inflammatory cells into the alveoli along with a reduction in total lung protein, which was in line with the results obtained by Feng et al. with bone marrow-derived MSC. The reduction of lung protein appears to be more significant in the CM-MSCs group as compared to the AD-MSC group, hence the conclusion that CM-MSC therapy has a superior effect. It has been difficult to fully explain the basic process by which AD-MSC wields this beneficial impact. Nevertheless, our findings indicated a significant decrease in neutrophils infiltrating into lung tissue, showing that AD-MSC had an anti-inflammatory effect on it. Neutrophils are activated by inflammatory mediators and migrate to the lungs, where they produce neutrophil extracellular traps (NETs), ROS, and proteases.[40] This in turn promotes epithelial and endothelial damage besides alveolar edema and exacerbation of the pro-inflammatory state.

AD-MSCs effect is thought to be due in part to their ability to reduce vascular permeability in turn reducing neutrophils influx to the lungs and suppressing inflammatory responses. This is an important finding, as neutrophil activation and migration have a substantial contribution to the onset and progress of acute lung injury.[41-44]

Numerous experiments have indicated that NO has a considerable contribution in both acute or chronic stages of toxicity caused by SM .[43,45] There is extensive evidence showing that SM can speed up the process of oxidative stress by increasing ROS production and reducing the ability to counteract oxidative damage and repair DNA. Human respiratory epithelial cells exposure to SM can also trigger off-release cytokines and inflammatory mediators (inducible NOS) leading to extensive damage.[46] There is a big link between exposure to sulfur mustard and oxidative stress as has

been proven in a study conducted in rats on how sulfur mustard affects the lungs.[47]

According to current research, NO levels in the peritoneal cavity of an acute CEES model showed a conspicuous decrease due to the injection of CM-MSC and AD-MSCs.[7] In the prevention of chemical-induced tissue injury, neutrophils and macrophages secrete ROS and NO into the tissues. Preventing these cells from recruitment and activation has been found to be effective in the locality of alleviating tissue damage. Also, different degrees of protection from SM-caused injuries have been offered by the NO synthase inhibitors and antioxidants.[48]

Likewise, in one study of a CEES-induced chronic model, treating with AD-MSCs effectively mitigated progressive histopathological changes in the lung and reduced the accumulation of alveolar macrophages. These findings highlight the therapeutic value of MSCs in combating SM-related inflammation and provide encouraging prospects for future treatments.[8,10] Inhalation of SM could cause severe airway damage.[9] Acute lung injury results from the leakage of fluid rich in protein into the area around blood vessels, due to the weakening of the barrier between the alveoli and cells that line them.[49] The study's model of lung injury from SM is characterized by these pathological changes; an influx of mononuclear cells, smooth muscle hyperplasia surrounding the airways, the presence of fibrin strands around blood vessels, edema, and chronic interstitial pneumonia in the lung tissue in conjunction with the escalated levels of expression of total protein were confirmed. However, treatment with AD-MSCs or CM-MSCs exhibited significant mitigation of histopathological changes, including the reduction of acute inflammation, hemorrhage, bronchiectasis, thickening of the basal membrane, and epithelial necrosis.

Our findings showed a significant reduction in protein levels in mice treated with CM-MSC and AD-MSC. A previous study showed that in the SM-induced acute mouse model), treating with bone marrow-derived MSC (intravenously) resulted in a reduction of BALF protein levels.[8] Zhang et al. showed that the levels of total protein in the BALF were decreased in the human adipose tissue-derived MSCs treated group compared to the control group. An increased concentration of protein in the BALF is indicative of cellular injury or increased endothelial and epithelial hyper-permeability.

The peritoneal cavity has a combination of immune cells that play an important role in monitoring visceral organs and ensuring the stability and balance of tissues (homeostasis) with essential functions.[50] Macrophages are the essential population in the peritoneal cavity that express high levels of surface markers, CD11b and F4/80.[51-53]

The plasticity of macrophages has made their roles in various diseases particularly interesting for developing new treatments .[54] These cells also regulate the homeostasis of the peritoneal cavity.[55] We showed that the injection of AD-MSCs not only decreased the inflammatory marker such as NO, but also caused a decrease in the PEMs. Supporting this evidence, we found that proinflammatory cytokines and anti-inflammatory cytokines were decreased in PEM culture supernatants in mice treated with AD-MSCs.

Our results suggest that MSC administration reduces the number of PEMs in the studied group compared to the control group. This decrease might be due to a shift in PEMs from a pro-inflammatory (M1) to an anti-inflammatory (M2) state. This change aligns with the known immunomodulatory properties of MSCs.

However, proving this issue requires more studies in the field of PEM subgroups. This study aligns with Agudelo et al.'s findings, where mice with thioglycolate-induced peritonitis receiving MVs, MSCs exhibited a significant reduction in PEMs.

Most previous studies explored the SM effect on pulmonary endothelial cell monolayers in a culture dish; the number of these cells was counted after exposing them to an SM analog and treating them with MSCs was our focus.

In the acute model, the administration of MSCs resulted in a significant decrease in the number of PEMs in comparison with a control group, aligning with the immunomodulatory effects of MSCs.

To summarize, our research showed that AD-MSCs have systemic therapeutic effects in treating injuries caused by SM in mice. These effects include reducing inflammation, modulating the immune system, and promoting tissue repair. AD-MSC treatment presents an encouraging and innovative method for addressing SM poisoning. However, additional research is necessary to gain a comprehensive understanding of the mechanisms underlying the therapeutic effects of AD-MSCs on SM-induced injuries.

STATEMENT OF ETHICS

This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC 1399.339).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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